UNIVERSITY OF TASMANIA

CAPILLARY ELECTROPHORESIS OF RIBOSOMAL RNA FOR CHARACTERISATION OF MICROBIAL COMMUNITIES

by

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Declaration

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Statement of Co-Authorship

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ABSTRACT

This thesis documents research on new capillary electrophoresis (CE) based rRNA fingerprint approaches for characterisation of low diversity microbial communities.

In the first body of work, an alternative approach for sieving polymer synthesis through reversible addition fragmentation chain transfer (RAFT) polymerisation is presented. Sieving polymer matrices are typically synthesised by conventional free radical polymerisation. This thesis describes the first synthesis of a high molecular weight poly(n,n-dimethylacrylamide) (PDMA) in which both the molar mass and polydispersity distribution were controlled by RAFT polymerisation. A multi-step chain extension is detailed and the physical properties and separation performance of DNA/RNA using this RAFT polymer are described.

The second body of work deals with the development of new approach for characterisation of microbial communities using CE. The new approach involves conformational separation of microbial 16S ribosomal RNA (rRNA) molecules containing the highly variable regions present in 16S rRNA. Single stranded conformation polymorphism (SSCP) is a separation technique based on the principle that for nucleic acid fragments of equal lengths, variation in sequences can affect nucleic acid folding and hence can be separated due to the difference in electrophoretic mobility. While CE DNA-SSCP has been commonly applied in clinical mutation diagnostic tests and studies of microbial diversity, CE rRNA-SSCP has yet to be demonstrated. In this work, an enzymatic based RNA-oligonucleotide cleavage method was employed to cleave the 16S rRNA (~1542 bases) to smaller fragments of similar length (~340 bases). This strategy uses a eubacterial ‘scissor’ probe to target and

hybridise highly conserved sites within the rRNA flanking highly variable regions (*e.g.* V1, V2 or V3). As rRNA is synthesised only by actively-growing cells, together with its role as the marker molecule for assigning sequences to genera and species, it can thus be used to correlate to the functioning members of microbial communities. Taking advantage of these unique properties, CE-rRNA-SSCP circumvents the need for polymerase chain reaction (PCR) amplification and retains the quantitative information regarding to the evenness of the microbial community that is important for ecological studies that were otherwise lost during PCR step. Compared to gel electrophoresis based approach, CE- rRNA SSCP significantly decreased the analysis time from 24 hours to 60 min and the use of a fluorescently labelled hybridisation probe for detection decreased the sample requirement by ten-fold. The combination of fast analysis time, low sample requirement and sensitive fluorescence detection makes CE-rRNA-SSCP an appealing new approach for characterising low diversity microbial communities.

The third body of work deals with the conception and development of a novel characterisation approach termed multiplex cleavage microbial community analysis (MCMCA), which is a potential method to simultaneously link the phylogeny of multiple groups of metabolically active microorganisms to their respective metabolic activity and relative abundance within a community. MCMCA utilizes the similar sequence-specific cleavage of rRNA molecules with oligonucleotides and RNase H employed in previous approach but differs by the use of multiple taxon specific probes selected to specifically cut the 16S rRNA into discrete fragments varying in length. The cleaved rRNA mixture is subsequently mixed with a fluorescently labelled locked nucleic acid (LNA) universal hybridisation probe and resolved using denaturing CE size separation. The feasibility of this rational is tested using model microbial strains,

followed by optimisation of the cleavage procedure to achieve multiplex cleavage in a model microbial community. This approach was then applied to characterise a hydrocarbon degrading enrichment community derived from soil.

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LIST OF ABBREVIATIONS

Acronym	Representation
A/T/C/G/U	adenine / thymine / cytosine / guanine / uracil
ARDRA	amplified ribosomal DNA-restriction analysis
BGE	background electrolyte
DMSO	dimethylsulfoxide
BRF	biased reptation with fluctuations
CAE	capillary array electrophoresis
cDNA	complimentary DNA
CE	capillary electrophoresis
CRP	controlled radical polymerisation
CSE	capillary sieving electrophoresis
DEPC	diethyl pyrocarbonate
DGGE/TGGE	denaturing or temperature gradient gel electrophoresis
DMA	dimethyl acrylamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EOF	electroosmotic flow
EtBr	ethidium bromid
FISH	fluorescence in situ hydridization
HEC	hydroxyethyl cellulose
HPMC	hydroxypropylmethyl cellulose
LB	Luria-Bertani

LIF	laser induced fluorescence
LNA	locked nucleic acid
LPA	linear polyacrylamide
MCMCA	multiplex cleavage microbial community analysis
Mn	number-average molecular weight
mRNA	messenger RNA
Mw	weight-average molecular weight
NanoSIMS	nanoscale secondary ion mass spectrometry
OD	optical density
OTU	operational taxonomic unit
PABTC	2-propionic acidyl butyl trithiocarbonate
PCR	polymerase chain reaction
PDI	polydispersity index
PDMA	poly-*n*,*n*-dimethyl acrylamide
PEO	polyethylene oxide
PFE/PFCE/PFGE	pulse field– gel/capillary electrophoresis
PHEA	polyhydroxyethyl acrylamide
PNA	peptode nucleic acid
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
RAFT	reversible addition fragmentation chain transfer
RDP	Ribosomal Databse Project
RNA	ribonucleic acid
rRNA	ribosomal RNA
SEC	size exclusion chromatography

ss/ds DNA	single/double stranded DNA
SSC	sequence specific cleavage
SSCP	single stranded conformation polymorphism
SSU	small subunit
TAPS	N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TBE	tris Borate EDTA
T_m	melting temperature
tRFLP	terminal restriction fragment length polymorphism
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TTE	Tris TAPS EDTA
μTAS	micrototal analysis systems

PREFACE

The importance of studying microbial biodiversity

Bacteria, archaea and viruses are the predominant form of life on the planet, yet the existence of bacteria is often overlooked in environmental and human health research and remains generally under-studied. It has been estimated that the 7000 identified species (as of 2008) of prokaryotes represent only 1-10% of all bacterial species, suggesting that we have only just begun to understand their diversity [1]. Microorganisms play critical roles in our world; they are vital in the functioning of all ecosystems including the human body, and central to the sustainable development of our environment owing to their roles in the cycling of the carbon, nitrogen, phosphate and other trace elements. Recently, there are also increasing in interest in studying human associated microbial communities, e.g. the Human Microbiome Project, as the microbial dysbioses (imbalance) have been implicated in a number of human diseases [2-4]. As such, it is important to study microbial diversity and, the more we understand the relationships and roles of the diverse organisms in the functioning of that system, the better we will understand the effects that human activities or lifestyles are exerting on microbial diversity and processes. This will enable the design of informed strategies to address pressing global issues such as anthropogenic climate change as well as coming up with treatments to cure microbial dybioses related diseases.

For many decades, microbial classification has been dependent on physical appearance and biochemical tests that require laborious cultivation and isolation of pure cultures

from the microbial community[1]. These approaches have little practical use as it was realised that typically less than 1% of the species present in a given environment can be cultured in the laboratory [6]. A further limitation is that due to the classification method, the microorganisms must be removed from their original environment. This results in the alteration of community structure through the provision of new selective conditions. Consequently, a new community structure evolves which may not be the best representation of the original structure.

An overview of culture independent molecular techniques for studying soil microbial communities.

During the last decade, the advent of molecular techniques have enabled microbial ecologists to develop new genetic fingerprinting methods that are independent of the culturability of unknown microorganisms and instead rely on the inherent genetic differences, particularly 16S ribosomal RNA (rRNA) genes, between every microorganism [7]. 16S rRNA genes are used extensively as a taxonomic marker for documentation of the evolutionary history and taxonomic assignment of individual organisms [8-12] because it encodes the small subunit (SSU) RNA (also known as 16S rRNA) that is ubiquitous in bacterial and archaeal genomes. It possesses highly conserved regions, which can be used for the construction of universal primers, and highly variable regions for identification of individual species [13]. Initial efforts to study microbial diversity relied on cloning isolated target 16S rRNA genes and then determining the nucleotide sequence. Although Sanger sequencing became a routine

[1] The concept of community ecology derived in plant and animal ecology. Communities are defined as multi-species assemblages, in which organisms live together in a contiguous environment and interact with each other. Refer to Ref [5] for a more detail definition.

procedure, sequencing thousands of clones is still a labour intensive, time consuming and expensive approach to examining microbial diversity, especially as it cannot provide information pertaining to the relative abundance[2] of microbial community.

Alternative techniques have been developed for the study of species richness (the number of different species, or diversity, represented in an ecological community,) and evenness (the relative amount number of each species compared to the total population in an environment) based on the physical separation of DNA fragments for each organism defined by species-specific 16S rRNA genes. These methods include denaturing or temperature gradient gel electrophoresis (DGGE & TGGE) [14] and terminal restriction fragment length polymorphism (tRFLP) [15, 16], amplified ribosomal DNA-restriction analysis (ARDRA) and single stranded conformation polymorphism (SSCP) [17].

Denaturing / Temperature Gradient Gel Electrophoresis (DGGE/TGGE)

DGGE and TGGE, are great in providing information about the community composition, which are useful in the rapid screening of multiple samples for distinguishing soil microbial communities. In DGGE/TGGE analysis, DNA fragments with the same length but different nucleotide sequences are separated [14, 18]. This separation for both techniques is achieved through the differences in mobility of polymerase chain reaction (PCR)-amplified DNA in polyacrylamide gels with a linear gradient of denaturant (e.g. urea) or temperature. As the amplified DNA strands migrate

[2] The relative abundance of microbial community refers to how common or rare a species is relative to other species in a microbial community.

in the sieving matrix, they began to dehybridised (melt) at particular melting domains and thus they become partially single-stranded. Partly denatured or fully denatured molecules stop migrating in the gel and DNA fragments occupy different positions in the gel according to their sequence composition and sequence variation. A guanine-cytosine clamp (GC rich sequence) attached to the 5′–end is used as a special primer to anchor the PCR fragments and prevent them from completely dissociating. DGGE/TGGE are sensitive methods to detect variation in 16S rRNA genes sequences. Well-separated bands can be excised from the gel, cloned and sequences for identification. However, their limitations range from being time consuming and difficult to reproduce due to gel-to gel variation, multiple bands could derived from single species due to micro heterogeneity, and limited resolution when applied to communities with high complexity whereby the profile may appear smeared to the huge number of bands.

Amplified Ribosomal DNA-Restriction Analysis (ARDRA)

ARDRA is a powerful tool for bacterial identification and classification at species level and it has been used to group and classify large sets of isolates and clones [19-21]. ARDRA generates restriction fragment profiles from the 16S rRNA gene amplicon of bacterial populations. After amplification, the amplicon is digested using tetracutter restriction enzymes and subsequently analysed on an automated DNA sequencing gel [22]. The restriction patterns data can then be compared with restriction analysis of rDNA sequences of known bacteria obtained using database sequences.

Single Stranded Conformation Polymorphism (SSCP)

Similar to DGGE/TGGE, detects sequence variations between different PCR amplicons normally derived from variable regions of the rDNA [17, 23]. Conventionally in SSCP one primer is fluorescently labelled at the 5′ end, and the phosphorylated strand of the PCR amplicons is selectively digested with lambda exonuclease. The intact strands are separated by electrophoresis under non- denaturing conditions (low temperature) in a polyacrylamide gel optimal for SSCP. The method is based on the differential intra-molecular folding of single-stranded DNA that is itself dependent upon DNA sequence variations. Thus, DNA secondary structure alters the electrophoretic mobility of the single-stranded PCR amplicons enabling them to be resolved. The reproducibility and discriminatory ability of the method is dependent on the fragment size and the position of the sequence variation within the fragment [17] and normally gives best results with fragments smaller than 400 basepair (bp). DNA-SSCP has also been successfully carried out in commercial capillary electrophoresis (CE) systems. SSCP has been used to differentiate between pure cultures of soil microorganisms and to distinguish community fingerprints of non- cultivated rhizosphere microbial communities from different plants [24, 25]. A limitation of the method, in addition to potential PCR bias, is that a single bacterial species may yield several bands due to the presence of several operons or more than one conformation of the single-stranded PCR amplicons.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP analysis is based on the restriction endonuclease digestion of fluorescent end-labelled PCR amplicons [26-29]. These PCR amplicons are derived from microbial community DNA using primers that complimentary to consensus sequences flanking

the variable regions in 16S rRNA genes. Both primers used for PCR amplification are labelled at the 5′ end with fluorescent dyes. After amplification, the amplicons are subjected to restriction enzyme digestion and separated either by gel or commercial CE system. The fluorescently labelled fragments are detected with a laser detector in an automated analyser and thus this technique only detects the “terminal” end labelled restriction fragments. Different soil microbial communities will exhibit distinct combinations of restriction sites because of the variation on the sequences of the gene that has been amplified, thus “fingerprints” profiles can be derived for particular assemblages of organisms. However, different DNA amounts may disturb the abundance and operation taxonomical unit (OTU; similar to species) in a T-RFLP profile. T-RFLP have been used to distinguish communities and to study community structure and dynamics in soils [30].

Fluorescence in situ hybridisation (FISH)

FISH is a technique that has been used since the early 90s [31]. FISH based methods have a more limited discovery capacity. Nevertheless they are useful in discovering species composition within specific parts of the community as well as important spatial information at the cellular level. In FISH based methods, phylogenetic oligonucleotide probes complementary to 16S and 23S rRNA are designed *in silico* by aligning and comparing sequences in rRNA databases. These phylogenetic probes are labelled with fluorophore and used for in situ detection of single cells in environmental samples by whole cell hybridisation. Finally, the visualisation of target microorganisms is achieved using epifluorescence microscopy [31, 32]. Like FISH, slot blot or membrane hybridisation methods use 16S rRNA oligonucleotide probe for detection of targeted

nucleic acid. Membrane hybridisation of community fingerprints with phylogenetic probes has proved to be particular used in studying and quantifying changes in community members [33-35].

A brief summary of advantages and disadvantages inherent to each method is summarised in Table 1.

Table 1 Molecular methods for soil microbial diversity studies. PCR polymerase chain reaction, DGGE denaturing gradient gel electrophoresis, TGGE temperature gradient gel electrophoresis, SSCP single-strand conformation polymorphism, T-RFLP terminal restriction fragment electrophoresis, ARDRA amplified ribosomal DNA restriction analysis, FISH fluorescence in situ hybridisation

Methods	Required PCR	Information and resolution	Strengths	Weaknesses
DGGE / TGGE	+	Genetic fingerprint of communities. Affiliation of predominant community members. Intermediate resolution	Bands can be excised, cloned and sequenced for identification	Time consuming; Complex communities may appear smeared due to a large number of bands; Gel-to-Gel reproducibility
DNA-SSCP	+	Genetic fingerprint of communities. Affiliation of predominant community members. Intermediate resolution	Community members can be identified, Screening of potential variations in sequences	Low to intermediate resolution; Insensitive to large fragments; Presence of several operons will complicate the separation resolution
T-RFLP	+	Community composition, relative abundance of numerically dominant community members. Intermediate resolution	Enables analyses of a wide array of microbes; Highly reproducible Easy to compare community structures between different samples	Artifacts might appear as false peaks Distinct sequences sharing a restriction site will result in one peak Unable to retrieve sequences
ARDRA	+	Genetic fingerprint of simple communities, populations or phylogenetic groups. Discrimination at lower taxonomic (species) levels. High resolution	Highly useful for detection of structural changes in simple microbial communities No special equipment required	Labour and time-intensive More applicable to environments with low complexity; Co-migrating bands from similar microbial groups
FISH	-	Detection and specific counting of metabolic active microorganisms. Intermediate resolution	Comparative analysis of community structure; Detection and identification of active cells. Direct phylogenetic information on community members	Auto-fluorescence of some microorganisms. Accuracy and reliability is highly dependent on specificity of probe(s); Required prior knowledge on targeted sequences
RNA Slot blot hybridisation	-	Phylogenetic identification of metabolic active community members. Intermediate resolution	Qualitative and quantitative analysis of metabolic active populations in communities. Phylogenetic information on active community members	Labour and time-intensive; Accuracy and reliability is highly dependent on specificity of probe(s); Required prior knowledge on targeted sequences

While these techniques have been applied to study species richness and evenness in a myriad of environments and increased our understanding of microbial diversity [14, 16, 17], they each possess inherent limitations. Most notably, PCR, which is the fundamental common characteristic of these molecular techniques (DGGE, T-RFLP, ARDRA, and DNA-SSCP but not FISH based methods), has been reported to introduce biases during the amplification of the initial target nucleic acid sequence. [36-41]. As a result, actual information regarding the abundance of each species in the microbial community is drastically altered during the amplification process. Following that, the separation of amplified 16S gene fragments is achieved using polyacrylamide gel electrophoresis (with exception of tRFLP), which is slow and laborious [42] and suffers from poor resolution [43]. Moreover, it is difficult to obtain quantitative results from the gel-staining visualisation process.

An important challenge in microbial ecology is the quantification of species richness and evenness in diversity studies, as well as the degree of metabolic involvement of taxa in functional studies. PCR-based methodologies can only be partially achieved the first aspect. Nevertheless, recently developed methods based on FISH with Raman spectroscopy (Raman-FISH) [44] and Nanoscale secondary ion mass spectrometry (NanoSIMS) [45, 46] have been reported for the study of microbial metabolism through tracking the incorporation of isotopically labelled substances. These techniques, however, lack the capacity for diversity discovery similar to the other methods. Furthermore, the current high cost of the specialised equipment prohibits their wide adoption. This poses a dilemma whereby to date there is no one single characterisation method that can provide species diversity, abundance and degree of metabolic activity

simultaneously. Combined methodological approaches to derive information on species diversity, abundance and function may be expensive, complex, time consuming and not widely accessible. Hence, acquiring a simple, cost effective and 'single method' approach to achieve this is an ongoing challenge in microbial ecology.

Scope of thesis

The goal of this PhD project was to address the methodological challenges facing microbial ecology by developing a new capillary electrophoresis based microbial community characterisation approach that aimed to be simple, cost effective and a 'single method' approach.

The first part of the thesis deals with the investigation of reversible addition fragmentation chain transfer (RAFT) as a potential synthesis strategy to synthesise a high molecular weight sieving polymer. Polymers are the fundamental component for high resolution separation of nucleic acids in CE The choice of the polymer (e.g. physical properties) dramatically influences separation performance, which is related to its potential applications. The use of RAFT for the synthesis of sieving polymer not only offers the ability to produce a well defined high molecular weight polymer for high resolution separation in CE but also serves to assess the broader potential in making novel co-polymers with defined segments with different properties applicable to other biopolymers analyses.

The second part of the thesis deals with the development of a capillary electrophoresis based rRNA fingerprinting approach to characterise microbial communities. The use of rRNA molecules directly, instead of the complementary DNA (cDNA), in microbial community fingerprints has the potential to yield information on species diversity and abundance simultaneously. This is because: 1) rRNA contains species-specific

sequences that enable taxonomic identification and 2) rRNA concentration in the environment is directly related with metabolic activity, growth and cell numbers. As such, diversity fingerprints derived directly from rRNA without the use on PCR should reflect the abundance and degree of metabolic activity in a system.

The third part of the thesis deals with the conceptions and development of a novel microbial community characterisation approach, namely multiplex cleavage microbial community analysis (MCMCA). The use of multiple group-specific probes in the RNase H cleavage reaction was demonstrated to have the potential to identify multiple targeted groups of metabolically active microorganisms and provide information regarding their relative abundance within the microbial community.

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Chapter 1

Capillary electrophoretic system of ribonucleic acid molecules

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Capillary electrophoretic system of ribonucleic acid molecules. Nai YH, Powell SM, Breadmore MC *Journal of Chromatography A*, 2012, vol. 1267, p. 2–9.

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Chapter 2

Sieving polymer synthesis by reversible addition fragmentation chain transfer Polymerization

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Sieving polymer synthesis by reversible addition fragmentation chain transfer Polymerization Nai YH, Jones RC, Breadmore MC *Electrophoresis*, 2013.

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Chapter 3

Capillary electrophoresis ribosomal RNA single-stranded conformation polymorphism: a new approach for characterization of low-diversity microbial communities.

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Capillary electrophoresis ribosomal RNA single-stranded conformation polymorphism: a new approach for characterization of low-diversity microbial communities. Nai YH, Zemb O, Gutierrez-Zamora M-L, Manefield M, Powell SM, Breadmore MC .*Analytical and Bioanalytical Chemistry*, 2012, vol. 404, nº 6-7, p. 1897-1906.

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4. MULTIPLEX CLEAVAGE MICROBIAL COMMUNITY ANALYSIS

4.1. Introduction

In the previous chapter, a novel CE based direct microbial rRNA fingerprint approach was demonstrated. Whilst the approach shown is useful for characterising low diversity microbial communities, microbial ecologists are still faced with the challenge of establishing links between the microbial community diversity and their metabolic activity. To date, the highest resolution nucleic acids separation method is based on size. If rRNA fragments of different size could be created then it would be possible to exploit the higher resolution separation to allow the ability to separate more species and provide a greater ability to characterise more complex samples. This chapter conceptually explores this concept termed multiplex cleavage microbial community analysis (MCMCA), as a potential method to address the aforementioned challenges to link microbial phylogeny to metabolic activity and relative abundance. MCMCA utilises the similar sequence-specific cleavage of community 16S rRNAs with oligonucleotides and RNase H employed in previous chapter but instead of using single universal probe to generate rRNA fragments of similar length, the cleavage reaction is multiplexed by inclusion of a set of microbial group-specific DNA probes that hybridise at different positions on the corresponding rRNA targets. This gives rise to discrete rRNA fragments varying in lengths based upon the RNase H cleavage (Figure 4-1). In this chapter, the feasibility of the methodology is evaluated and proof-of-concept for MCMCA is demonstrated using model microbial strains with DNA probes that specifically target a large proportion of Actinobacteria, the α, β and γ subdivisions of

Proteobacterial 16S rRNA. The resulting rRNA fragments are subsequently labelled with a fluorescently labelled hybridisation probe (specific to universally conserved bacterial 16S rRNA sequences) and resolved through denaturing sieving electrophoresis by CE. The relative abundance and /or metabolic activity of the multiple targeted bacterial groups can be characterised by simultaneously determining the signal intensity from corresponding rRNA fragments. Finally, this approach was then applied to characterise the development of an enrichment of hydrocarbon degraders derived from soil.

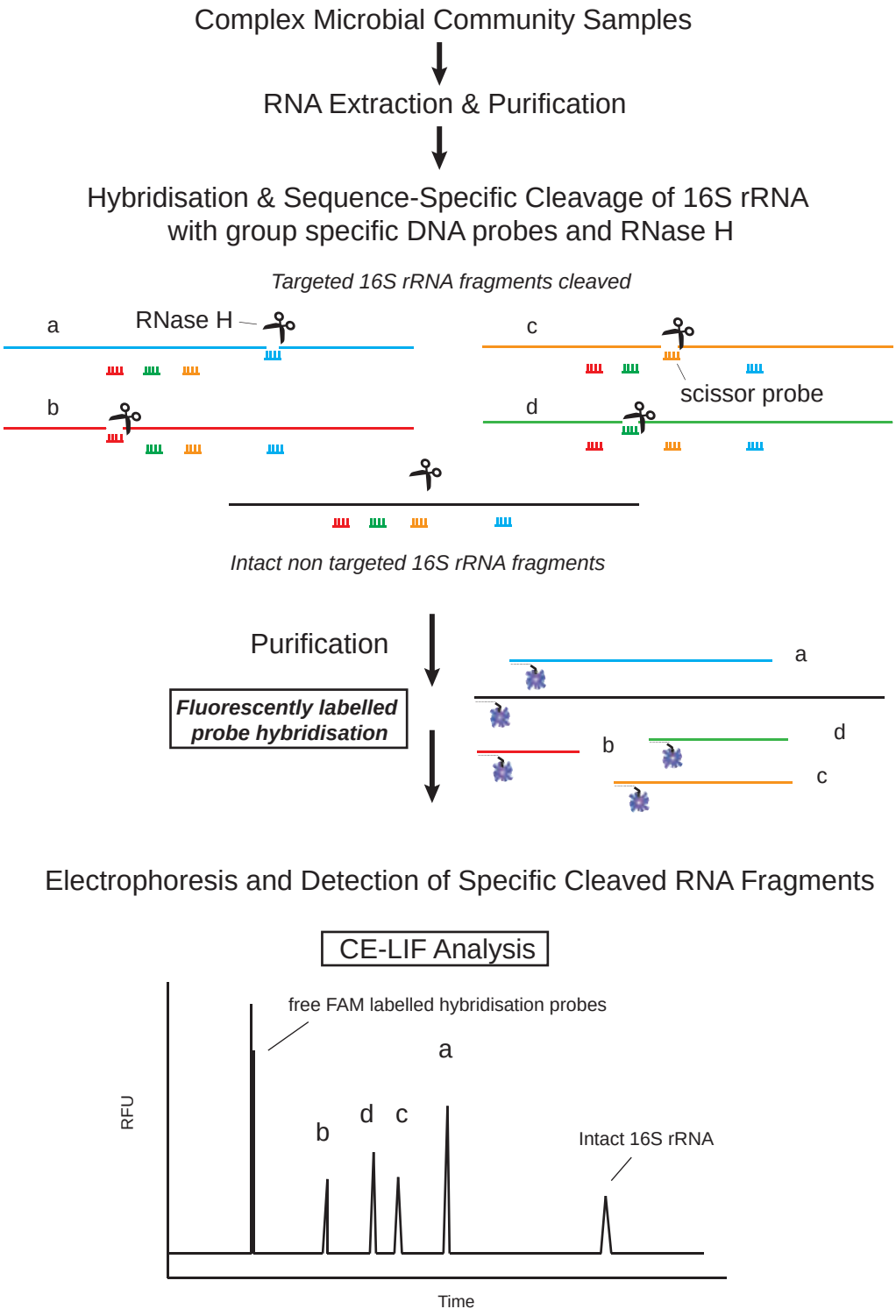

Figure 4-1 Schematic representation of multiplex cleavage microbial community analysis (MCMCA) by CE-LIF.

4.2. Experimental

4.2.1. Materials

All chemical reagents were either molecular biology or analytical reagent grade obtained from Sigma Aldrich (Sydney, Australia) unless stated otherwise. All buffers and solutions for the cleavage reaction were prepared in molecular biology grade nuclease-free water (Qiagen, Hilden, Germany). Background electrolytes and solutions were prepared in diethyl pyrocarbonate (DEPC) treated Milli-Q water (Millipore, Bedford, MA, U.S.A.). The sieving polymer used in this study was synthesised in house (described in Chapter 3).

4.2.2. Bacterial strains, environmental soil sample and culture conditions

Six microbial strains - *Sulfitobacter sp.* (Class α-Proteobacteria), *Chromobacterium violaceum* (Class β-Proteobacteria), *Pseudomonas aeruginosa* (Class γ-Proteobacteria), *Brevibacterium sp.* (Phylum Actinobacteria), *Bacillus polymyxa* (Phylum Firmicutes) and *Flavobacterium sp.* (Phylum Bacteroidetes) were used in this work as a model microbial community throughout the optimisation experiments. These microbial strains were obtained from the culture collection of the Food Safety Centre, from the University of Tasmania, Australia. To prepare total RNA from individual bacterial strains, cultures were grown individually in their respective agar media and, when necessary, in liquid media at conditions summarised in Table 4-1 and were harvested during the exponential phase. In the hydrocarbon degrader enrichment study, an environmental soil sample was collected from a household garden in Hobart, Tasmania, Australia. For the primary enrichment culture, 10 g soil was added into a presterilised

250 mL erlenmeyer flask containing 90 ml sterile Bushnell-Haas broth (BD Difco™) containing cyclohexamide (Oxoid, Thermo Fischer Scientific) at 0.5 µg/L to inhibit fungal growth and 1 % v/v kerosene (carbon source). After 3 days incubation at 25 ºC with shaking at 150 rpm, 5 ml of this primary enrichment culture was subsequently subcultured to a secondary enrichment flask containing fresh 95 mL Bushnell-Haas broth containing 0.5 µg/L cyclohexamide and 1% v/v kerosene and incubated as above.

Table 4-1 Bacterial strains used as model microbes in this study.

Microbial strain	**Classification**	**Growth medium and temperature**
Sulfitobacter sp.	Class α- Proteobacteria	Marine Broth, 25 ºC
Chromobacterium violeceum	Class β- Proteobacteria	Luria-Bertani broth, 37 ºC
Pseudomonas aeruginosa	Class γ- Proteobacteria	Luria-Bertani broth, 37 ºC
Bacillus polymyxa	Phylum Firmicutes	Luria-Bertani broth, 37 ºC
Brevibacterium sp.	Phylym Actinobacteria	Tryptic Soy Broth, 25 ºC
Flavobacterium sp.	Phylum Bacteriodetes	Nutrient broth, 25 ºC

4.2.3. Total RNA extraction

Total RNA extraction from pure cultures and the secondary hydrocarbon degrader enrichment culture were carried out using Norgen Biotek Total RNA mini extraction kit (Thorold, Ontario, Canada) according to the manufacturer's protocol. For the primary hydrocarbon degrader enrichment culture, total RNA extraction was carried out using Norgen Biotek Soil RNA extraction kit (Thorold, Ontario, Canada) according to the manufacturer's protocol for the removal of humic acids from soil. All RNA samples were eluted in elution solution (nuclease free water) and quantified on a Nanodrop 8000 (Thermo Fisher Scientific, Massachusetts, USA). Total RNA was used directly for cleavage reaction.

4.2.4. Sequence specific cleavage of rRNA with RNase H

Similar to Chapter 3, RNA was subjected to sequence specific cleavage (SSC) using RNase H (Ambion, Life Technologies) and scissor probes based on a previously published protocol [1, 2] with slight modifications.

For a 25 μL reaction, an aliquot of RNA suspension (containing 800 ng total RNA for single probe hybridisation-cleavage; and up to 2 μg total RNA for multiplex hybridisation-cleavage) was incubated at 70 ºC for 5 min then chilled on ice. The RNA suspension was then mixed with 1.25 μL of hybridisation buffer (375 mM Tris [tris(hydroxymethyl)aminomethane] HCl [pH 7.5], 15 mM EDTA (ethylenediaminetetraacetic acid), 375 mM NaCl), 3.75 μL deionised formamide and 2 μL of 100 μM scissor probe (Table 4-2) (For CE-rRNA SSCP, EUB342 probe was used as per Chapter 3). An appropriate amount of nuclease-free water was added to

bring the mixture volume to 18.75 µL. The mixture was subsequently heated at 95 ºC for 1 min and then maintained at an appropriate hybridisation and cleavage temperature (40 - 60 ºC). Following that, the cleavage reaction was initiated by the addition of 6.25 µL of pre-heated enzyme solution (25 mM Tris HCl, 40 mM $MgCl_2$, 25 mM NaCl, 4 mM dithiothreitol [DTT], 120 µg/mL of bovine serum albumin, 0.02 U/µL of RNase H) to the mixture and was incubated for 15 min. To terminate the reaction, 50 µL of stop solution (0.45 M sodium acetate [pH7.0] and 15 mM EDTA) was added to the reaction mixture. RNA was precipitated by addition of 190 µL of chilled ethanol and centrifugation at 14,000 rpm for 15 min at 4 ºC. Supernatant was carefully discarded and the pellet was dissolved in nuclease free water and subjected to further analysis.

Table 4-2 DNA scissors probes used for the evaluation of Phyla- / Class specific cleavage of 16S rRNA with model microbial strain total RNA. Model microbial strains used in this study include, *Sulfitobacter sp.* (Alphaproteobacteria), *Chromobacterium violaceum* (Betaproteobacteria) and *Pseudomonas aeruginosa* (γ-Proteobacteria), *Brevibacterium sp.* (Actinobacteria), *Bacillus polymyxa* (Firmicutes) and *Flavobacterium sp.* (Bacteroidetes)

Probe Name	Target Group	Sequence (5' - 3')	*Escherichia coli* position	Probe length	T_m (ºC)	G + C (%)	Specificity[3]	References
ALF682R	Class α-Proteobacteria	GAA TTT CAC CTC TAC ACTSG	682-701	20	49.7	40	94.30%	[3]
Alf685R	Class α-Proteobacteria	TCT ACG RAT TTC ACC YCT AC	685-704	20	47.7-52	40	81.80%	[4]
BETA359R *	Class β-Proteobacteria	CCC ATT GTC CAA AAT TCC CC	359-378	20	51.8	50	88.80%	[5]
BET680	Class β Proteobacteria	TCA CTG CTA CAC GYG	680-699	15	43	53.3	70.50%	[4]
Gamma871R	Class γ-Proteobacteria	AGC TGA CGA CAA CCA TGC AC	871-891	20	53.8	55	99.10%	[5]
Gamma395R *	Class γ-Proteobacteria	TTC ACA CAC GCG GCA TKG	395-412	18	50.3-52.6	55.6	89.50%	[5]
γ1202R	Class γ-Proteobacteria	CGT AAG GGC CAT GAT G	1202-1217	16	46	56.3	85.50%	[3]
Firm1060R	Phylum Firmicutes	AGC TGA CGA CAA CCA TGC AC	1041-1060	20	53.8	55	75.50%	[6]
Firm350f R *	Phylum Firmicutes	GAA GAT TCC CYA CTG CTG CC	350-367	20	53.8-55.9	55	97.30%	[5]
LGC353b	Phylum Firmicutes	GCG GAA GAT TCC CTA CTG C	353 - 371	19	49	44.4	99.90%	[7]
ACT235R *	Phylum Actinobacteria	CAA CAA GCT GAT AGG CCG CG	235-254	20	56	60	99.00%	[8]
ACT920R *	Phylum Actinobacteria	TAG CCT TGC GGC CGT A	920-935	16	48.5	62.5	91.80%	[3]
CFB560	Phylum Cytophaga-Flexibacter-Bacteroides	WCC CTT TAA ACC CAR T	560-575	16	38.3-40	37.5	95.60%	[9]
cfb967R	Phylum Bacteroidetes	GGT AAG GTT CCT CGC GTA T	967-985	19	51.1	52.6	96.70%	[3]
EUB342	Domain *Bacteria*	ACT GCT GCC TCC CGT AGG	341-358	18	54.9	66.7	87%	Chapter 3

* Reverse complement of published forward primer.

4.2.5. Fluorescence detection of rRNA with locked nucleic acid enhanced hybridisation probe

Similar to chapter 3, the conserved region of the 16S rRNA gene (*Escherichia coli* [*E. coli*] position 7-27) was used for designing the fluorescent hybridisation probe. However, the hybridisation probe (27R-Flc) used in the previous chapter was enhanced with locked nucleic acid (LNA) (FAM-27R-LNA, 5' CTG AGC CAK GAT CAA ACT CT 3'). The new probe, FAM-27R-LNA, has an elevated melting temperature T_m of 87-88 ºC compared to 62 ºC previously. The 5' end of the LNA probe was labelled with 6-carboxyfluorescein (6-FAM) and was custom synthesised by Exiqon (Vedbaek, Denmark).

4.2.6. Instrumentation

Electrophoresis of RNA fragments were performed using either an Agilent 2100 Bioanalyzer with the RNA 6000 Nano kit (Agilent Technologies, CA, USA) or a P/ACE MDQ Capillary Electrophoresis System instrument (Beckman-Coulter, CA, USA) equipped with 488 nm laser. For electrophoresis with the Agilent 2100 Bioanalyzer, RNA samples and microchip were prepared according to manufacturer's instructions. The signal intensities of individual band/peak(s) in the analysis were determined with the Agilent 2100 Bioanalyzer Software. For electrophoresis on P/ACE MDQ CE system, experiments were conducted using a single fused-silica capillary of 50 μm i.d. obtained from Polymicro Technologies (Phoenix, AZ, USA) with a total length of 50 cm (effective length to the detector 40 cm). New capillary was preconditioned with 1M NaOH for 5 min, water for 5 min and then filled with sieving matrix in background electrolyte using a pressure of 85

psi for 10 min. Separation of RNA was accomplished at 50 ºC using a sieving buffer consisted of 5% w/w PDMA (450 kDa) in background electrolyte of 4 M urea (final concentration), 44.5 mM TAPS, 44.5 mM Tris and 1 mM EDTA at pH 8.5. Each analysis began with flushing the capillary with sieving matrix for 5 min, followed by a pre-injection plug of Milli-Q for 5 s at 5 psi. Samples were prepared by combining 3 μL of the rRNA sample, 1 μL of fluorescently labelled hybridisation probe (1 μM FAM-27R-LNA in Tris-HCl EDTA, pH 8.0) and 16 μL deionised formamide. DNA ladder used in this study was synthesised in house using custom fluorescein labelled primers and pCR-TOPO plasmid sequence (see Thesis Appendix). The DNA ladder sample consisted of 18 μL deionised formamide and 2 μL DNA ladder, heated to 95 ºC for 5 min then rapidly chilled on ice before injection. The sample was electrokinetically injected at reversed polarity 10 kV (200 V/cm) for 100 s (5 s for DNA ladder) and separated at reversed polarity 10 kV (200 V/cm). rRNA fragments were detected by laser-induced fluorescence (LIF) with the fluorescently labelled hybridisation probe. CE-LIF data was collected and analysed using the 32 Karat software version 8 provided with the CE instrument.

4.3. Results and Discussion

4.3.1. Proof-of-concept of MCMCA

The proof-of-concept of MCMCA had the following three stages. Firstly, candidate probes were selected on the basis of their specificity towards target groups and hybridisation site in which the resulting rRNA fragments can be later separated by electrophoresis. In this work, 16S rRNA from the following strains belonging to Actinobacteria, Firmicutes, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria and Bacteroidetes were used as a model microbial community to test the feasibility of the proposed approach. In the second step, the specificity of each scissor probe was investigated by performing cleavage on target strain 16S rRNA followed by non-target strains at a range of stringencies. Furthermore, the denaturing CE separation parameters were optimised to resolve rRNA fragments generated by multiplex cleavage reaction. Finally, the proposed approach was applied to the characterisation of the enrichment of hydrocarbon degrading microbes from soil.

4.3.1.1. A newly designed microbial characterisation approach

RNase H is an endonuclease that specifically recognises a DNA:RNA hybrid structures [2]. Uyeno and co-workers have exploited the RNase H enzymatic ability to cleave bacterial rRNA with a suite of taxonomic scissor probes to study various microbial ecosystems [10-12]. However, in spite of its usefulness, the method is still considered laborious and time consuming in practice, particularly as the cleavage reaction is carried out with one scissor probe at a time, and sample preparation quickly becomes a strenuous process especially when comprehensive set of scissor probes is used. To precisely and simultaneously evaluate the abundance and activity

of selected groups of microbes in a complex community, a multiplex cleavage approach (Figure 4-1) capable of simultaneously cleavage of multiple 16S rRNA with specific scissor probes in a single reaction should be explored and developed.

4.3.1.2. Probe considerations

Table 4-2 shows a list of candidate probes documented in the literature that were developed for PCR assays in environmental microbiology that may be used directly as scissor probes in the RNase H cleavage process. These probes were highly specific and covered a large proportion of Actinobacteria, Firmicutes, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria and Bacteroidetes 16S rRNA. However, they were shown to be specific at different temperatures and discrete target regions. Therefore, it was important to find common reaction conditions to carry out hybridisation and cleavage in the same thermocycler. To demonstrate the concept of MCMCA, the probes ACT235R, BET359R, G395R, Alf682R, G871R, CFB976R and F1060R were selected as candidate scissor probes that upon RNase H digestion should result in cleaved rRNA fragments with discrete lengths of ~230 (ACT235R), 350 (BET359R), 370 (G395R), 600 (Alf682R), 870 (G871R), 960 (CFB976R) and 1050 (F1060R) nt (Figure 4-2 and Table 4-2). (Note: hybridised positions vary between organisms as the probe's denoted position corresponded to position of the *E. coli* 16S rRNA gene sequence).

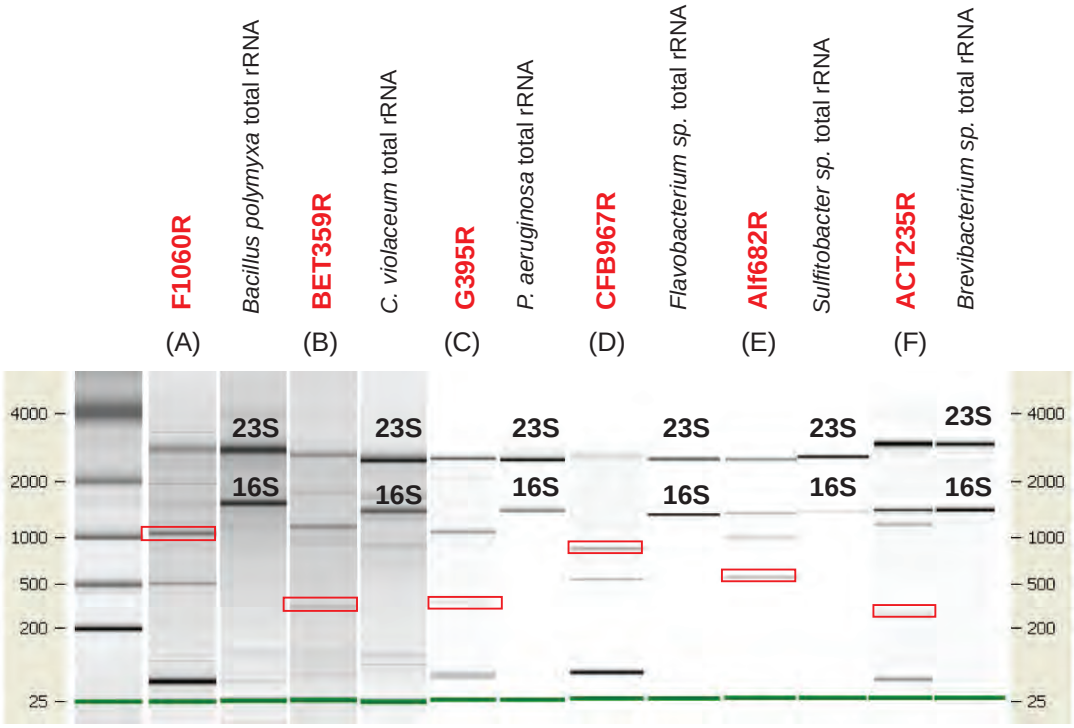

Figure 4-2 Gel like images of electropherograms of model strain RNA cleaved with the corresponding candidate probe and respective total RNA, as resolved by the Agilent 2100 Bioanalyzer with an RNA 6000 nano kit. Lane (A) F1060R - Phylum Firmicutes -*B. polymyxa*; (B) BET359R- Class β- Proteobacteria - *C. violaceum*; Lane (C) G395R - Class γ- Proteobacteria - *P. aeruginosa* (D) CFB967R - Phylum Bacteriodetes - *Flavobacterium sp.*; (E) Alf682R - Class α- Proteobacteria - *Sulfitobacter sp.*; (F) ACT235R - Phylum Actinobacteria - *Brevibacterium sp.*.

4.3.1.3. Scissor Probes evaluation and optimisation of the reaction conditions for the multiplex cleavage of rRNA

The temperature used for hybridisation and subsequent cleavage plays an important role in affecting the cleavage reaction [1]. For the evaluation of candidate probes, both hybridisation and cleavage were simultaneously performed at the same temperature from 40 up to 60°C under the defined conditions (800 ng of total RNA, 1 µM of scissor probe, hybridisation and cleavage for 15 min). The upper

temperature limit of both hybridisation and cleavage was set at 60 ºC as non-specific cleavage has been reported above this even in the absence of RNase H and the oligonucleotides, and was hypothesized to be caused by the physiochemical degradation of RNA at higher temperatures [1]. After specific cleavage, the 16S RNA was digested into two fragments, as demonstrated in Figure 4-3. The cleavage efficiency (percentage of digested 16S rRNA) of the probe was calculated with equation (Eq. 4.1) defined in Ref. [1] using the peak areas obtained from the Bioanalyzer electropherogram, whereby,

$$\%\ digested\ 16S\ rRNA = \frac{A_{fragment\ 1} + A_{fragment\ 2}}{A_{fragment\ 1} + A_{fragment\ 2} + A_{16S}} \times 100\% \qquad \text{(Eq. 4.1)}$$

$A_{fragment\ 1}$ is the peak area of cleaved rRNA fragment 1, $A_{fragment\ 2}$ is the peak area of cleaved rRNA fragment 2 and A_{16S} is the peak area of intact 16 rRNA.

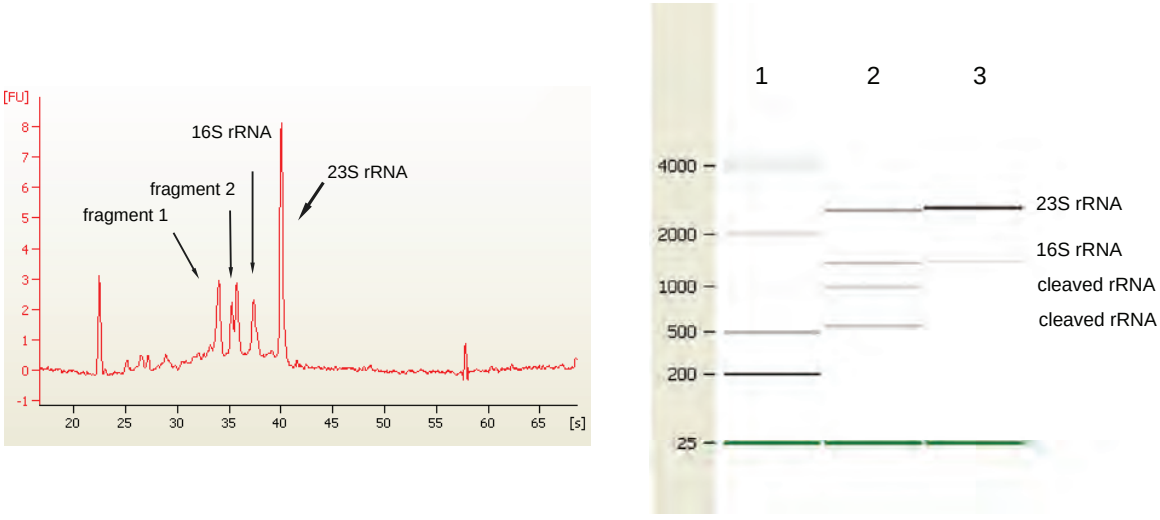

Figure 4-3 Left panel, electropherogram of *Sulfitobacter sp.* RNA cleaved with the Alf682R probe at 50 ºC, as resolved by the Agilent 2100 Bioanalyzer with an RNA 6000 nano kit. A gel-like image of the electropherogram produced by Bioanalyzer 2100 software is also shown in the right panel; lane 1, RNA 6000 ladder marker; lane 2, digested *Sulfitobacter sp.* RNA fragments; lane 3, *Sulfitobacter sp.* RNA negative control.

4.3.1.4. Modification of Alf682R probe

All scissor probes with exception of Alf682R have neutral G+C percentages (G+C% ~ 50-60%). Previous reports [1, 13] have shown the G + C content and the length of the probe play significant roles in contributing to the DNA-RNA duplex stability which is crucial for the activation of RNase H. As shown in Figure 4-4, Alf682R ([G+C] % =40 %, targeting position approx. 580 up to 610) could only partially digest *Sulfitobacter sp.* 16S rRNA across all hybridisation and cleavage temperatures examined in the range of 40 – 60 ºC. The moderate cleavage efficiency of Alf682R (65-79 %) across the studied temperature range indicated that the DNA-RNA duplex formation between the scissor probe and rRNA was unstable under these reaction conditions, possibly due to its low G + C content (Table 4-2).

To overcome this, the possibility of improving the Alf682R probe hybridisation stability with the inclusion of locked nucleic acids (LNA) was investigated. LNA are ribonucleotides containing a methylene bridge that connects the 2'-oxygen of ribose with 4'-carbo (see reviews [14, 15]). Inclusion of LNA in the design of antisense oligonucleotides was first described by Wengel and co-workers [16] and Imanishi and co-workers [17]. Inclusion of LNA has been shown to greatly improve the affinity of oligonucleotides for complementary sequences thus leading to a more stable heteroduplex as well as increasing the melting temperature (T_m) by several degrees [14].

However, the position of the LNA in the LNA/DNA mixed-oligonucleotides is crucial to ensure the highest stability for binding and successful activation of RNase H cleavage of the target RNA [18]. A chimeric LNA-DNA-LNA oligonucleotide to contain a stretch of seven or eight DNA monomers, with three LNAs at the 3'- and

5'-ends, has been shown to be necessary for RNase H activation and also offers the highest stability. Using these guidelines, the Alf682 probe was modified with LNA sequence, 5' GAATTTCACCTCTACACTSG 3' (LNA-Alf682R, underlined sequence being LNA sequences), and has a new T_m of 64-71 ºC compared to 49.7 ºC for the original sequence. As shown in Figure 4-4, the cleavage efficiency of Alf682R was greatly improved with the inclusion of LNA sequence and complete cleavage of *Sulfitobacter sp.* 16S rRNA across the hybridisation and cleavage temperature range.

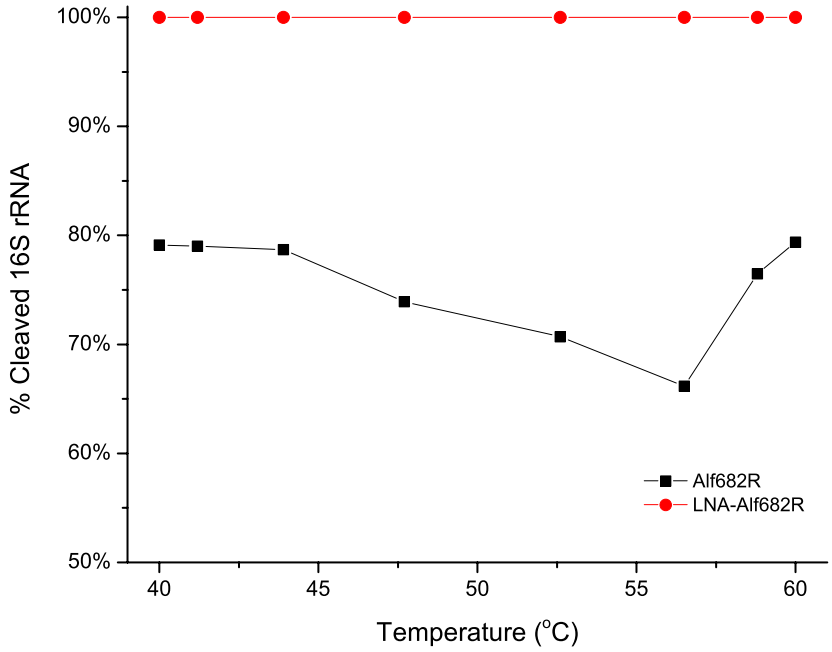

Figure 4-4 Temperature dependence of the rRNA cleavage reaction with the Alf682R and LNA-Alf682R probes. Percentages of cleaved 16S rRNA in the total 16S rRNA were directly estimated based on the peak areas of intact and cleaved 16S rRNA fragments in the electropherograms.

4.3.1.5. Determination of common reaction temperature for hybridisation and cleavage

Sequence specific cleavage of the chosen candidate probes was observed with varying cleavage efficiency over the studied temperature range from 40 - 60°C. Complete sequence-specific cleavage of the corresponding model strain rRNA molecules were found at hybridisation and cleavage temperatures ranging from 53 to 60°C (Figure 4-5) with ACT235, G395R, LNA-Alf682R, F1060R and CFB967R. The cleavage efficiencies of probes G871R and BET359R however, gradually diminished with increasing reaction temperature from 53 ºC and 57.5 ºC, respectively. Furthermore, some of the scissor probes (F1060R, CFB967R and G871R) exhibited lower rates of rRNA fragmentation at low temperatures (40 to 50°C) than at higher temperatures (50 to 60°C). This phenomenon was believed to be due the obstruction of probe binding by the secondary and tertiary conformations of rRNA at lower temperatures [13, 19]. Based on the cleavage curves shown in Figure 4-5, a hybridisation and cleavage temperature of 53 ºC was selected as the most appropriate condition to provide complete cleavage of all of the models.

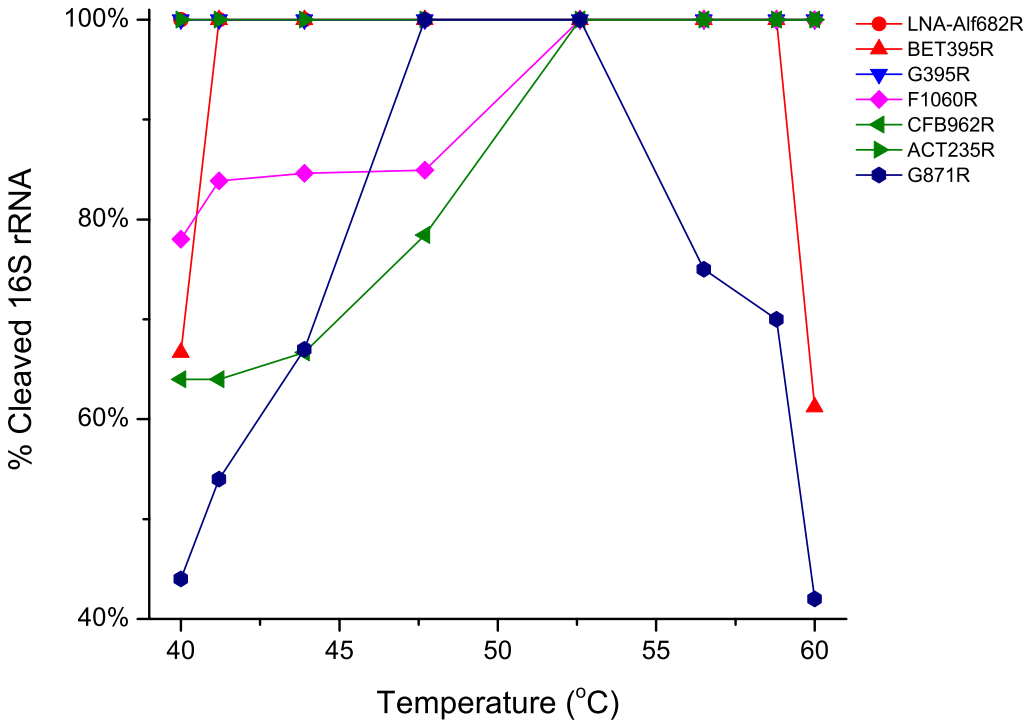

Figure 4-5 Temperature dependence of the rRNA cleavage reaction with the selected candidate probes. Percentages of cleaved 16S rRNA in the total 16S rRNA were directly estimated based on the peak areas of intact and cleaved 16S rRNA fragments in the electropherograms, and the percentages were plotted with the hybridisation and cleavage temperatures at which the respective reactions were performed.

4.3.1.6. CE analysis of Initial Multiplex cleavage rRNA fragments

Using the reaction condition of 53 ºC for both hybridisation and cleavage temperatures, a preliminary multiplex cleavage was carried out with defined conditions (800 ng of total RNA from each model strain, combined probe set with each probe concentration at 1 µM, hybridisation and subsequent cleavage at 53 ºC for 15 min). The resulting rRNA fragments were labelled with the fluorescent hybridisation probe and subsequently analysed on the CE platform as illustrated in Figure 4-1, which will be discussed in detail below.

The CE analysis parameters studied in previous chapters, including choice of sieving polymer, polymer concentration, field strength, urea concentration, background electrolyte, injection time, and detection strategy, were adapted for the denaturing size separation of rRNA fragments. The analysis was carried out with field strength of 200 V/cm using a sieving matrix consists of 5% w/w PDMA (450 kDa) and in background electrolyte (BGE) of 5M urea, 44.5 mM TAPS, 44.5 mM TRIS and 1 mM EDTA (pH 8.5) in an uncoated capillary.

Previously, it was necessary to maintain the analysis temperature at 18 ºC for the conformation separation in the CE-rRNA SSCP approach. To perform accurate size separation, the temperature parameter can be optimised to improve denaturing effects to minimise the effect of RNA secondary and tertiary conformation at low temperatures as discussed in Chapter 1 [20]. Nevertheless in Chapter 3, it was demonstrated that the analyses performed at higher temperatures with 5 M urea (denaturant) were detrimental to hybridisation detection due denaturing effect which caused the fluorescent hybridisation probe to dehybridise from the rRNA fragments.

To resolve the detection issue at high analysis temperatures, a LNA-enhanced fluorescently labelled hybridisation probe, FAM-27R-LNA, was designed as the new rRNA detection hybridisation probe for CE-MCMCA. Ten locked nucleic acids were included into the oligonucleotide FAM-27R-LNA sequence (see *section* 4.2.5), and this increased the melting temperature T_m to 87-88 ºC as compared to T_m = 62 ºC for the 27R-Flc probe, used in Chapter 3. FAM-27R-LNA was expected to have greater tolerance towards urea and temperature compared to 27R-Flc. As shown in Figure 4-6, fluorescence signals from rRNA fragments were still detected at 50 ºC under 5 M urea denaturing condition.

To successfully demonstrate the multiplex cleavage process, a mixture of individually cleaved model strain rRNA fragments was combined using the corresponding candidate probes under the defined conditions (see above) and is shown in Figure 4-6 [a]. The sizes of the RNA fragments corresponded well with the expected size range for the candidate probes with the exception of G871R, where by the resulting rRNA fragment was shown to have a similar size to the fragment cleaved by F1060R leading to a co-migrated peak at approximately 1040 nt. This was confirmed by using the 'Probe Match' tool on Ribosomal Database Project (RDP) [21]. This showed that the G871R probe is complementary to *P. aeruginosa* 16S rRNA sequence at approximated 1015-1030 nt positions (varies depending on strains) and not at the expected 870 nt. As a result, G871R was removed from the candidate probe set and replaced by G395R for subsequent work.

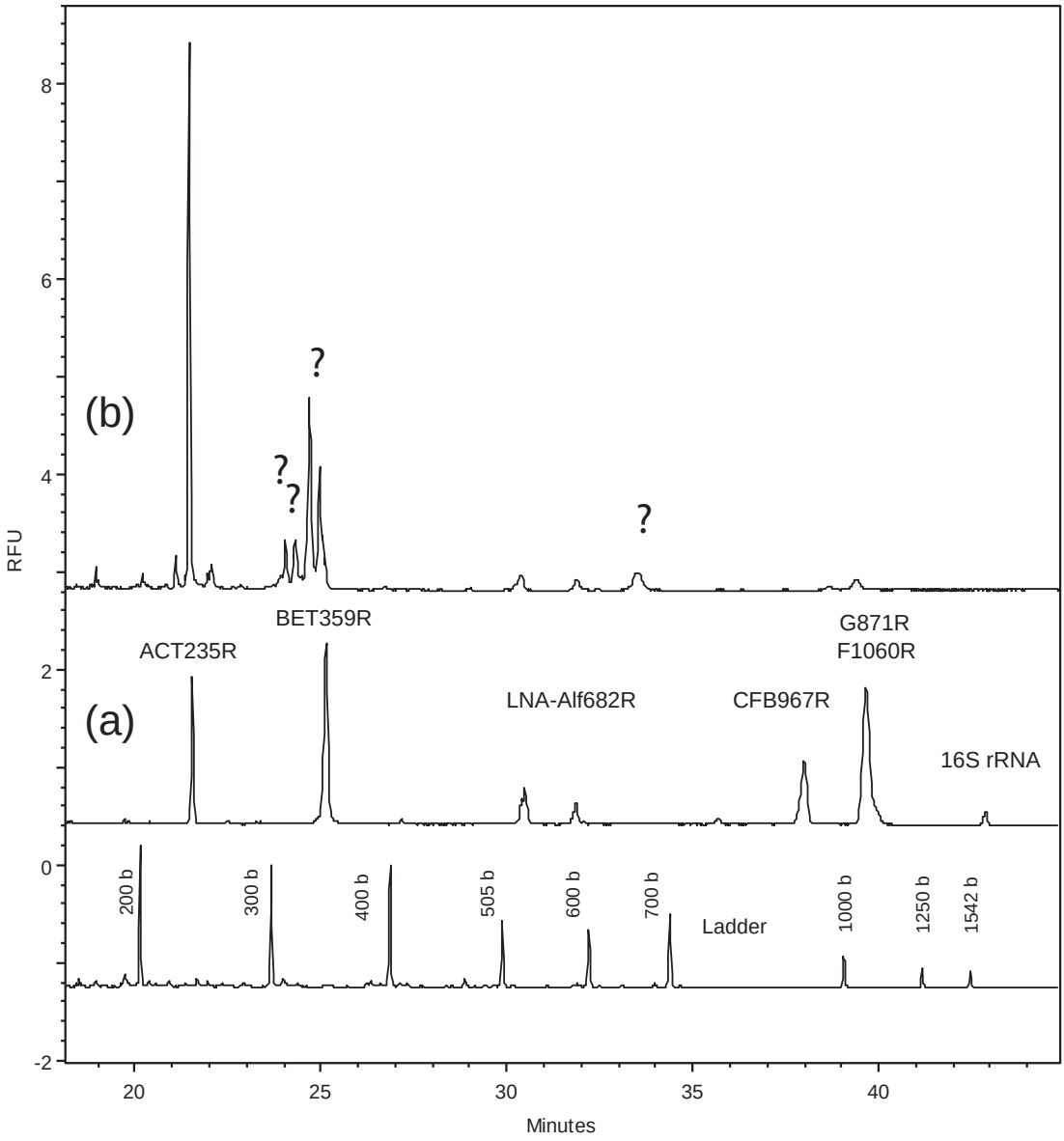

Figure 4-6 Electropherograms of (a) artificial mixture of individually cleaved model strain RNA cleaved with 1 μM of corresponding scissor probes at 53 ºC (b) artificial microbial community cleaved with ACT235R, BET359R, LNA-Alf682R, CFB967R, F1060R and G871R probe (each at 1 μM) at 53 ºC for 15 min; bottom trace, in house single stranded DNA ladder. Electrophoresis conditions: 5% w/w PDMA in 5 M urea 44.5 mM TAPS 44.5 mM Tris and 1 mM EDTA (pH 8.5), 50 μm x 50.2 cm fused silica capillary, Injection procedure: a deionised water plug was introduced hydrodynamically (12 psi for 0.5 min) followed by reversed polarity 10 kV, 100 s injection of rRNA fragments in deionised formamide. Electrophoresis of RNA was carried out at reversed polarity 200 V/cm at temperature of 50 ºC.

Figure 4-6 (b) shows the trace of rRNA fragments from a multiplex cleavage reaction at 53 ºC. The electropherogram trace is distinctly different from the mixture of individually cleaved model strains rRNA (Figure 4-6 [a])— suggesting some of the scissor probes were hybridised to non-target 16S rRNA which resulted in additional rRNA fragments after cleavage. To elucidate the extent of the scissor probe cross-reactivity toward non-target 16S rRNA, each probe (ACT235R, BET359R, LNA-Alf682R, CFB967R, F1060R and G395R) was systematically evaluated by subjecting non-target model strain 16S RNA to hybridisation with the scissor probe and RNase H cleavage at 53 ºC. It was discovered that each probe exhibited varying degrees of cross reactivity toward non-target 16S rRNAs. Examples of the nonspecific hybridisation on non-target 16S rRNA, are shown in the Figure 4-7, the LNA-Alf682R probe for Class α- Proteobacteria cleaved non-target 16S rRNA of Class γ- Proteobacteria (*P. aeruginosa*) and Phylum Firmicutes (*B. polymyxa*); F1060R probe for Phylum Firmicutes cleaved Phylum Bacteriodetes (*Flavobacterium sp.*) and Class γ- Proteobacteria (*P. aeruginosa*); BET359R probe for Class β- Proteobacteria cleaved Class α & γ- Proteobacteria *(Sulfitobacter sp* & *P. aeruginosa*)*;* G395R probe for Class γ- Proteobacteria cleaved Phylum Firmicutes (*B. polymyxa*) and Class α (*Sulfitobacter sp.*). In particular, the result for LNA-Alf682R was interesting as the non-modified probe, Alf682R, exhibited no hybridisation reactivity (and RNase H cleavage) toward the two other non-target model strains (*P. aeruginosa* and *B. polymyxa*) over the studied temperature range from 50 - 60°C (Figure 4-7 [d]). This implies that whilst the modification of Alf682R with LNA improves its cleavage efficiency towards *Sulfitobacteria sp.* RNA, the enhancement in DNA-RNA duplex stability could be problematic with non-target 16S rRNA that has only a few mismatched bases. These observations indicated that

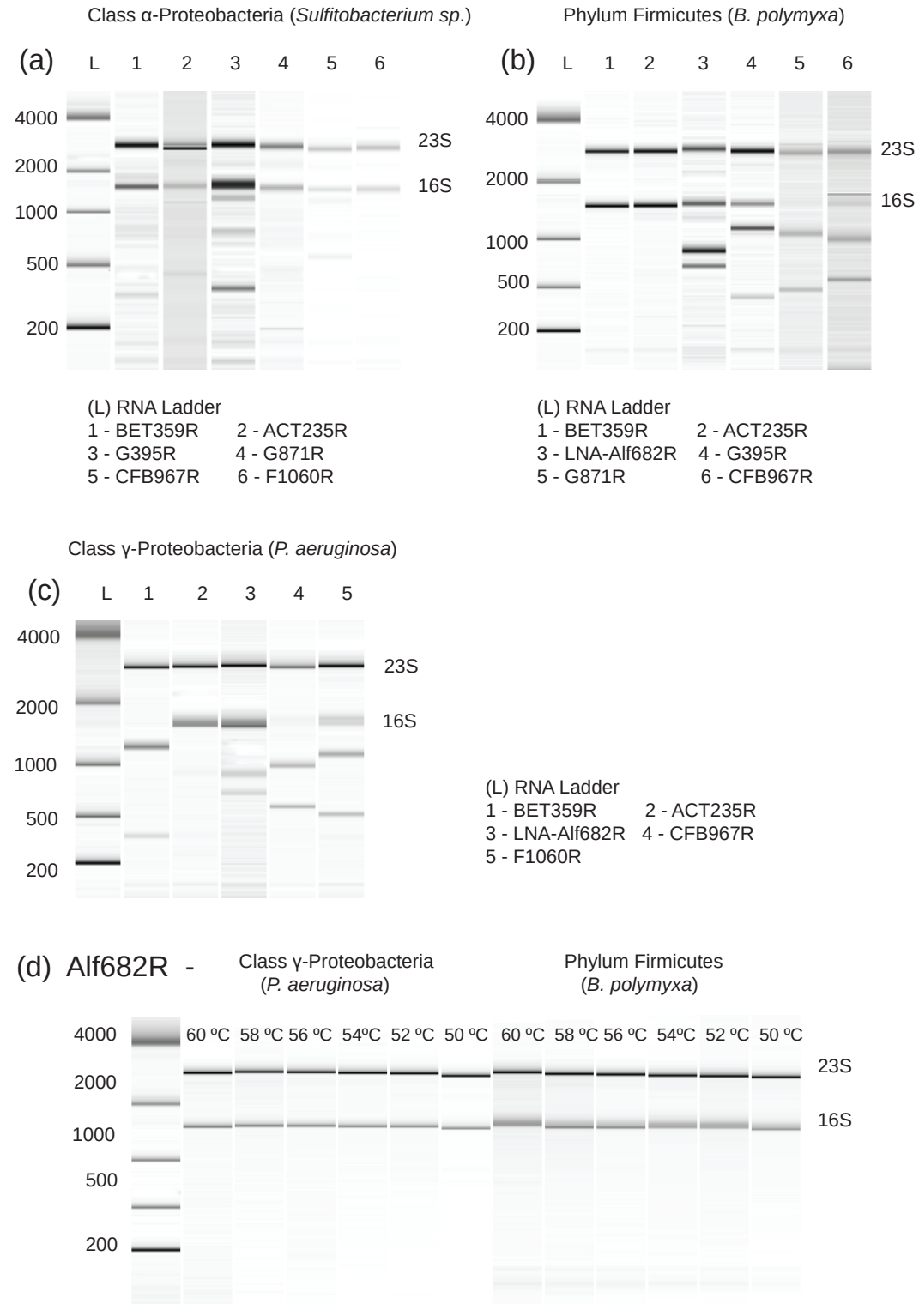

Figure 4-7 Gel-like images of electropherograms of (a) Class α- Proteobacteria - *Sulfitobacter sp*.; (b) Phylum Firmicutes -*B. polymyxa*; (c) Class γ- Proteobacteria - *P. aeruginosa*, RNA cleaved with 1 µM of non-target scissor probes at 53 ºC; (d) Class γ- Proteobacteria - *P. aeruginosa* and Phylum Firmicutes -*B. polymyxa* RNA cleaved with 1 µM non-modified Alf682R probe at 50 -60 ºC.

the reaction condition for multiplex hybridisation-cleavage thus far may not have sufficient stringency and requires further optimisation but is realistic given the ability to develop suitable PCR primers for single nucleotide polymorphisms.

4.3.1.7. Adjustment of hybridisation stringency using temperature and formamide

Optimisation of the temperature and addition of formamide have been shown to be effective measures to improve hybridisation stringency in *fluorescence in situ hybridisation* (FISH) [22] and sequence specific cleavage by RNase H [12]. Previous reports [1, 22, 23] have shown that each hybridisation probe usually has its optimal formamide concentration and hybridisation temperature. To overcome the obstacles of 6 different scissor probes hybridising to non-target 16S rRNA, optimisation of common multiplex reaction conditions, including hybridisation-cleavage temperature and formamide concentration, was carried out with the LNA-Alf682 probe and *P. aeruginosa* and *B. polymyxa*. The probe dissociation curves of individual probes are shown in Figure 4-8; using these curves, the optimum reaction temperature and formamide concentration were determined at which the scissor probe would specifically cleave only the targeted 16S rRNA (*Sulfitobacterium sp.*). As shown in Figure 4-8 (a), *P. aeruginosa* 16S rRNA was no longer targeted by LNA-Alf682R probe at a formamide concentration of 10% (at 50 ºC). However under these conditions, *B. polymyxa* 16S RNA was still cleaved (Figure 4-8 [b]) and much more stringent conditions, 57.5 ºC with 15 % formamide, were required to prevent the cleavage of *B. polymyxa*. Unfortunately, the cleavage efficiency of LNA-Alf682R probe toward *Sulfitobacterium sp.* 16S rRNA has decreased to 60% under such

stringent conditions. These conditions were subsequently used as the new conditions for the probe re-evaluation. Similar to the previous process, each probe (ACT235R, BET359R, LNA-Alf682R, CFB967R, F1060R and G395R) was re-evaluated by subjecting target and non-targeted model strain 16S RNA to cleavage reaction (57.5 ºC and 15 % formamide) with the scissor probes. Gel like electropherograms of the evaluation process are shown in Figure 4-9. *Sulfitobacterium sp.* RNA, as shown in Figure 4-9(A) was the only RNA that was specifically but partially cleaved by LNA-Alf682R under the new reaction condition, a similar trend was observed for *Brevibacterium sp.* and ACT235R probe (Figure 4-9 [F]). Both BET359R (Figure 4-9 [B]) and G395R (Figure 4-9 [C]) probes are specific for their respective target 16S rRNA but still cleave *Sulfitobacter sp.* RNA. Given the fact that α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria belong in the same phylum group, BET359R (Figure 4-9 [B]) hybridisation towards *Sulfitobacter sp.* RNA may be due to the high sequence similarity between these subclasses and could be solved by the design of a new probe in future studies. Unfortunately, probe CFB967R (Figure 4-9 [D]) could no longer cleave *Flavobacterium sp.* RNA due to the instability of the DNA-RNA duplex under the new reaction conditions. Interestingly, as shown in (Figure 4-9 [E]), probe F1060R has failed to cleave *B. polymyxa* RNA but remained effective at cleaving non-target 16S RNA of *Brevibacterium sp.*. These results revealed that these two oligonucleotide probes are unsuitable for the present method and would require further modification and optimisation in future studies. As a result, probes CFB967R and F1060R were removed from the candidate probe set and the remaining four probes, ACT235R, BET359R, G395R and LNA-Alf682R were used for further multiplex cleavage experiments.

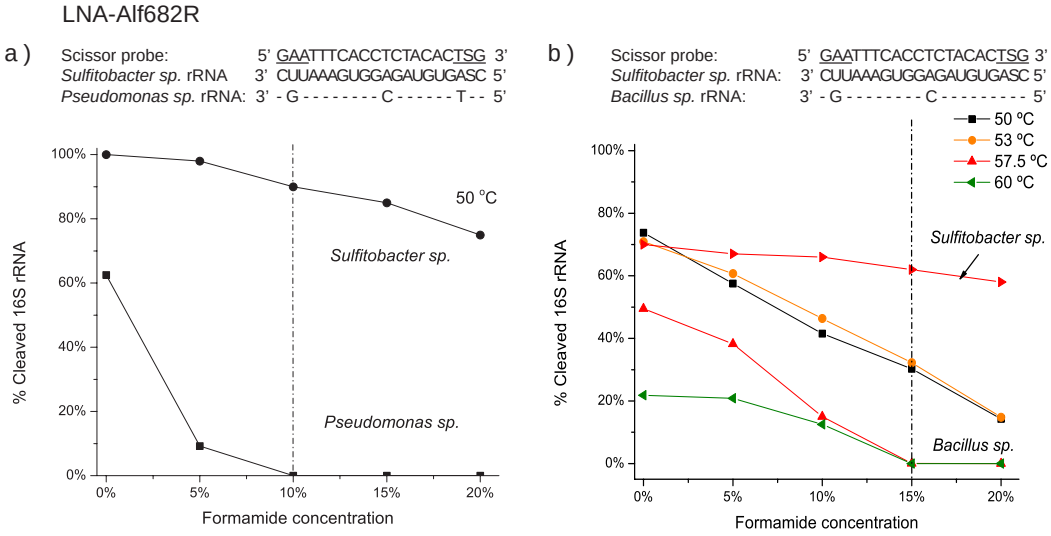

Figure 4-8 Probe dissociation curves of LNA-Alf682 scissor probe under increasingly stringent hybridisation and cleavage conditions for the cleavage reaction. For each graph, data points indicate percentage of cleaved 16S rRNA in the total 16S rRNA estimated from Agilent 2100 Bioanalyzer electrophoregram of RNA fragment (see above). In all of the experiments, 16S rRNAs of *Sulfitobacter sp.*, *P. aeruginosa* and *B. polymyxa*, were used for the digestion. LNA-Alf682 probe sequence and the corresponding target sequences of the 16S rRNA of the tested organisms are indicated; dashes in the non-target rRNA sequences represent nucleotides identical to those of the target rRNA sequences. The vertical dotted lines indicate the optimum formamide concentration.

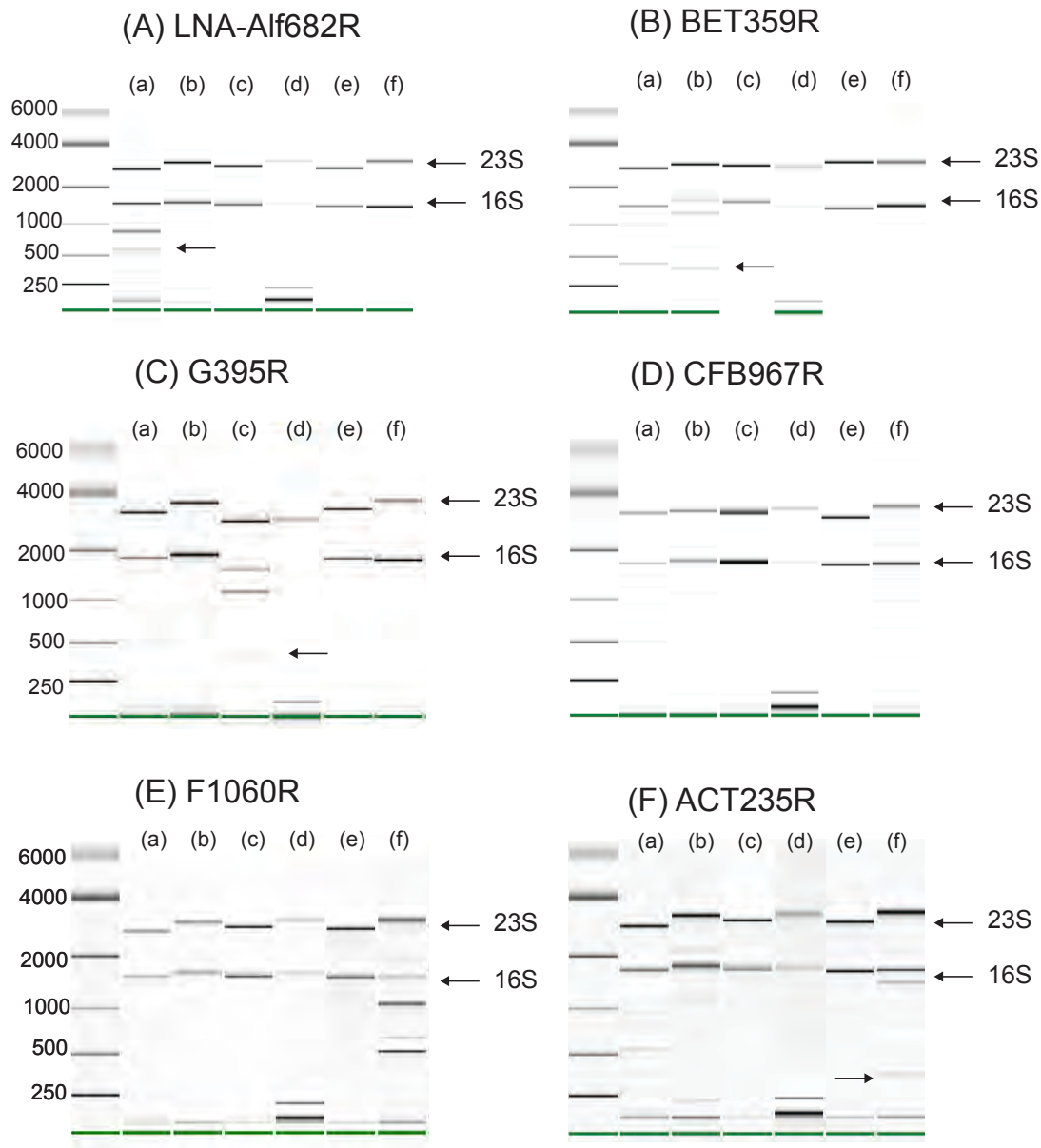

Figure 4-9 Gel-like images of electropherograms of model strain RNA cleaved with 1µM of (A) LNA-Alf682R; (B) BET359R; (C) G395R; (D) CFB967R; (E) F1060R and (F) ACT235R, with 15 % formamide at 57.5 ºC. Lane (a) Class α- Proteobacteria - *Sulfitobacter sp*.; lane (b) Class β- Proteobacteria - *C. violaceum*; lane (c) Class γ- Proteobacteria - *P. aeruginosa* (d) Phylum Bacteriodetes - *Flavobacterium sp*.; lane (e) Phylum Firmicutes - *B. polymyxa*; lane (f) Phylum Actinobacteria - *Brevibacterium sp*.; Arrows indicate the cleaved rRNA fragments; these rRNA fragments possess the conserved region complementary to fluorescent hybridisation probes sequence.

4.3.1.8. Effect of the scissor probe concentration in the hybridisation-cleavage buffer on the cleavage efficiency

From Figure 4-9, cleavage efficiencies of scissor probes LNA-Alf682R, BET359R, G395R and ACT235R were clearly compromised with the elevated hybridisation-cleavage temperature and inclusion of formamide. Under the new defined conditions (800 ng of rRNA and hybridisation-cleavage at 57.5°C with 15 % formamide), specific but partial cleavage of 16S rRNA was observed in hybridisation - cleavage buffer (25 μL) containing 1 μM of the scissor probe. These findings suggest the stability of DNA-RNA duplex between the scissor probes and the rRNA under these conditions was diminished by the elevated reaction temperature and denaturing condition. However, as shown in Figure 4-10, the collective specific cleavage efficiency of 16S rRNA could be improved by increasing the probe concentration in the hybridisation-cleavage buffer. BET359R, G395R and ACT235R probes demonstrated drastic improvement on their respective cleavage efficiencies from 10-40 % at 0.5 μM and up to above 50% at 4 μM, respectively. Interestingly, the cleavage efficiency of LNA-Alf682R experienced minimal changes throughout the concentration range remaining at 60% (i.e. probe-RNA duplex is stabilised) yet presently the author could not offer any possible explanation to this phenomenon. This could be addressed by further investigation examining whether or not the observed duplex stabilisation was caused by the LNA in LNA-Alf682R [18].

To prevent the slight but non-specific cleavage of the RNA that was previously reported [1] at higher concentrations (equivalent to 5 μM in 25 μL) of the probe in of hybridisation-cleavage buffer, 4 μM probe concentration was selected as the optimised concentration in the multiplex cleavage reaction.

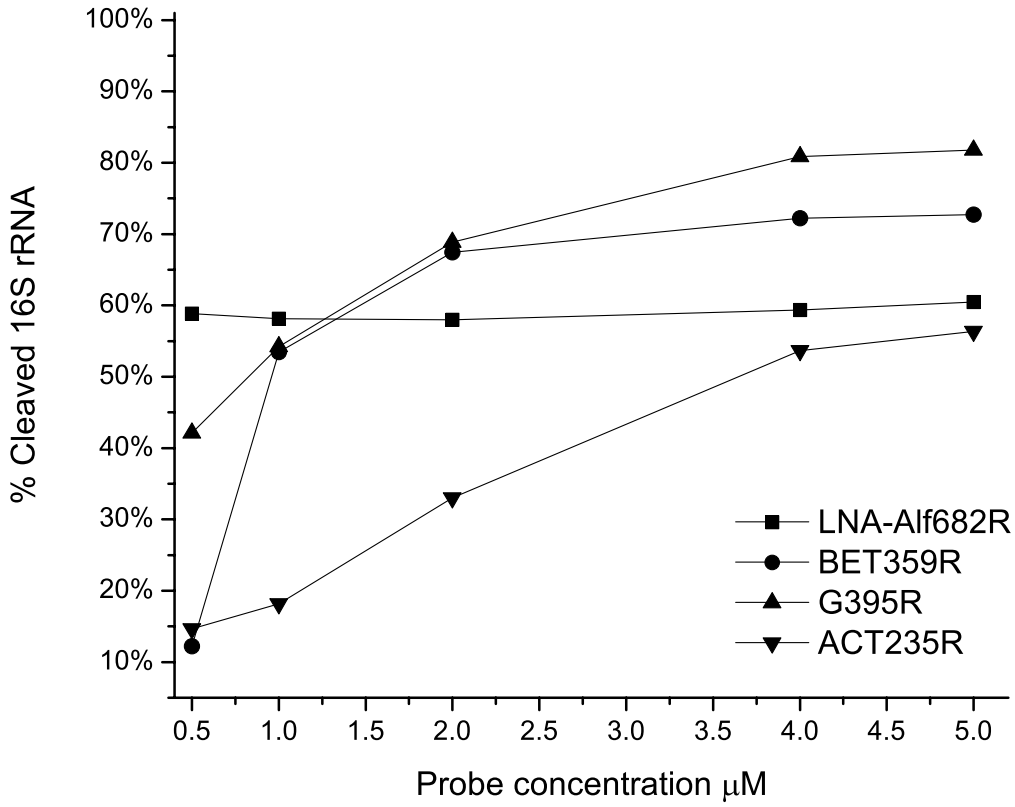

Figure 4-10 Effect of scissor probe concentration on cleavage efficiency. Data points indicate percentages of cleaved 16S rRNA in the total 16S rRNA estimated from Agilent Bioanalyzer electropherograms of RNA fragments. Hybridisation-cleavage (57.5°C with 15 % formamide for 15 min) of model strain RNAs was performed with different probe concentrations.

4.3.1.9. Optimisation of CE-LIF size separation of RNA

As discussed Chapter 1 and *section* 4.3.1.6, the addition of denaturant is necessary for accurate size separation of RNA as the denaturant minimises the effect of RNA secondary and tertiary conformation formation which significantly altered the RNA electrophoretic mobilities and enhanced the separation resolution. In addition, analysis temperature can also be optimised to improve the denaturing effects. The sizes of the rRNA fragments resulting from multiplex cleavage using scissor probes ACT235R BET359R, G395R, and LNA-Alf682 at the optimal conditions (2.4 μg of artificial microbial community: *Brevibacterium sp*. RNA, *C. violaceum* RNA, *P. aeruginosa* RNA and *Sulfitobacter sp.* RNA [800 ng, respectively] and hybridisation-cleavage at 57.5°C with 15 % formamide) were estimated to be approximately 230 nt, 335 nt, 360 nt and 600 nt (and 370-390 nt due to BET359, Figure 4-9[B]), respectively. To separate all these fragments with adequate resolution, size separation was carried out with electrophoresis conditions as described in *Section* 4.3.1.6 and temperatures of 25, 35, 40 and 50 ºC to examine the influence of temperature on MCMCA separation. As shown in Figure 4-11, rRNA fragments from G395R - *P. aeruginosa* and BET359-*Sulfitobacter sp*. co-migrated at 25 ºC. G395R - *P. aeruginosa* gradually separated from the BET359-*Sulfitobacter sp.* peak with increasing the separation temperature. Together with the denaturing condition of 5 mol/L urea, the resolution was optimal at 40 ºC as it provided adequate baseline separation for BET359 - *C. violaceum*, G395R and BET359R-*Sulfitobacter sp.* Thus analysis temperature of 40 ºC (was chosen along with other conditions (5% w/w PDMA in 44.5 mM TRIS 44.5 mM TAPS 1 mM EDTA (pH 8.45), field strength of 200 V/cm) to be the optimal conditions for MCMCA. The

electropherograms of multiplex cleavage products of the artificial microbial community and individual model strains are shown in Figure 4-12.

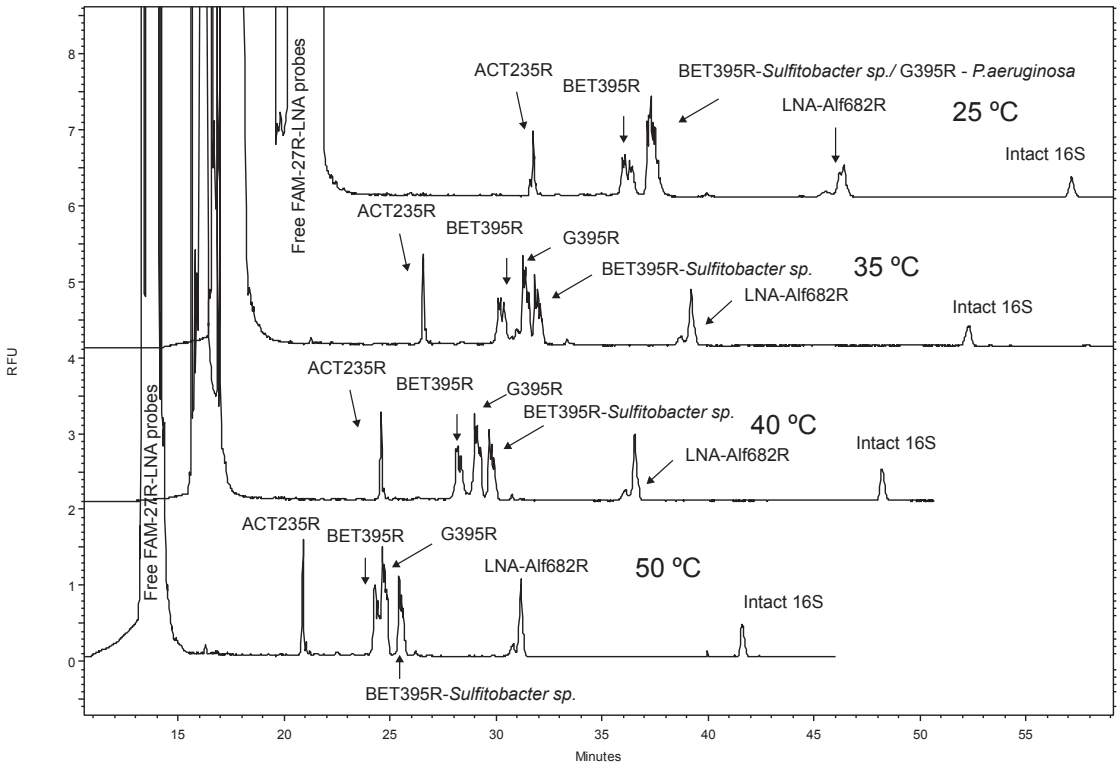

Figure 4-11 Effect of analysis temperatures on CE analysis of rRNA fragments of 4 model strains RNA cleaved with ACT235R, BET359R, G395R and LNA-Alf682R probe (each at 4 μM) at 57.5 ºC and 15 % formamide. Electrophoregrams shown illustrate the effect of temperatures ranging from 25 – 50 ºC on RNA separation resolution. Other electrophoresis conditions as described in Figure 4-6.

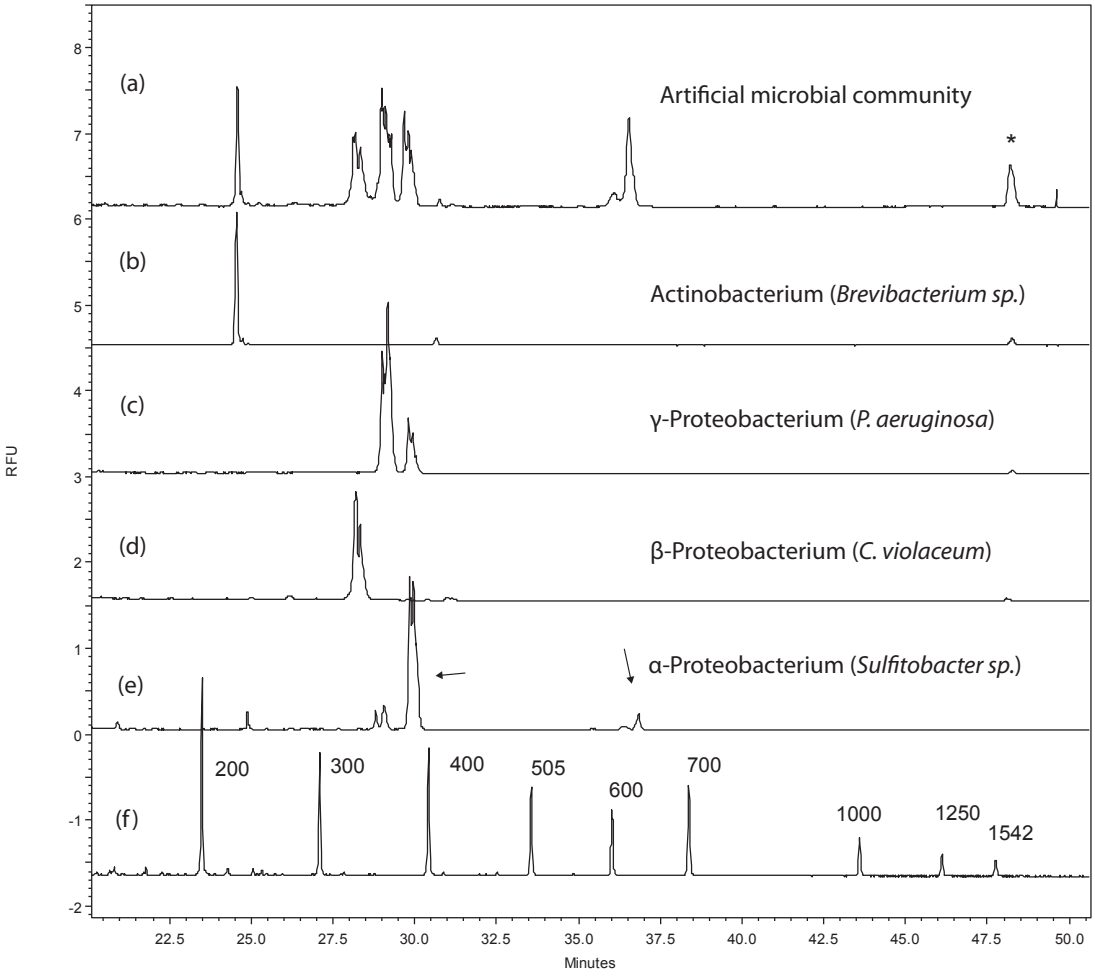

Figure 4-12 Electrophoregrams showing separations of resulting RNA fragments from multiplex cleavage reaction using scissor probes ACT235R, BET359R, G395R and LNA-Alf682R probe (each at 4 μM) on (a) artificial microbial community of 4 model strains RNA (b) *Brevibacterium sp*. RNA; (c) *P. aeruginosa* RNA; (d) *C. violaceum* RNA; (e) *Sulfitobacter sp.* RNA. Single stranded DNA marker (200 - 1542 nt) is present as size reference guide. Electrophoresis conditions: 5% w/w PDMA in 5 M urea 44.5 mM TAPS 44.5 mM Tris and 1 mM EDTA (pH 8.5), 50 μm x 50.2 cm fused silica capillary, Injection procedure: a deionised water plug was introduced hydrodynamically (12 psi for 0.5 min) followed by reversed polarity 10kV, 100 s injection of rRNA fragments in deionised formamide. Electrophoresis of RNA was carried out at reversed polarity 200 V/cm at temperature of 40 ºC.

4.3.2. Application of MCMCA and CE-rRNA SSCP on hydrocarbon degrading community enriched from soil

Bacteria play the central role in hydrocarbon degradation [24] as they possess the ability to utilise hydrocarbons to satisfy their cell growth and energy needs. There is an increased interest in promoting biological methods in the process of cleaning oil-polluted sites as bioremediation methods are considered to be a cost-effective approach to clean up crude oil spills compared to conventional remediation methods which include physical removal of contaminated materials and use of chemicals [25]. A large number of studies have identified various microbial groups, especially γ-Proteobacteria [26-29], α-Proteobacteria [26, 27, 29] and to some degree β-Proteobacteria and Actinobacteria [26, 29], as effective degraders of hydrocarbons in natural environments. Several strains from these classes have been isolated based on their ability to metabolize various carbon sources, such as aliphatic and polycyclic aromatic compounds and their chlorinated derivatives.

To demonstrate a potential application of the developed method, MCMCA was applied in conjunction with CE-rRNA SSCP analysis to study the dynamics and relative metabolic activities of several microbial groups capable of utilising hydrocarbon as an energy source within enrichment cultures. As shown in Figure 4-13 (a), community diversity fingerprints were successfully generated by CE-rRNA SSCP analysis which corresponded to shifts in metabolic activity during the enrichment process. The fingerprint profiles showed a gradual decrease in signal peak width over the primary and secondary enrichment cultures. This observation may indicate that the community diversity was gradually becoming less diverse and

the hydrocarbon treatment favored bacteria that can degrade hydrocarbons resulting in major shifts in the microbial communities within the enrichment.

To find out which microbial groups are abundant and active during hydrocarbon degradation, the application of MCMCA with probes ACT235R, BET359R, G395R and LNA-Alf682R on the RNA extracts allowed the characterisation of the targeted bacterial group dynamics within the enrichment cultures. As shown in Figure 4-13 (b), 16S rRNA of α-Proteobacteria and γ-Proteobacteria were detected in primary enrichment culture and later became the dominant groups in the secondary enrichment culture. These findings are in good agreement with other reports on metagenomic analyses of hydrocarbon bioremediation environments [26-29]. The 16S rRNA from β-Proteobacteria was only briefly detected in the enrichment cultures. The author was unable to provide any experimental explanation to account for the diminishing rRNA signal from β-Proteobacteria, although it was speculated to cause by the competition with other microbes present in the enrichment culture.

Actinobacteria are usually the dominant microbial group in soil [30, 31], but interestingly they were not detected by MCMCA in this study. This result does not conclusively indicate the absence of Actinobacteria. Another possible explanation could be due to the fact that they grow slowly [26] in contrast to γ-Proteobacteria [32] which could explain the predominant rRNA signals from the latter group during early enrichment stages.

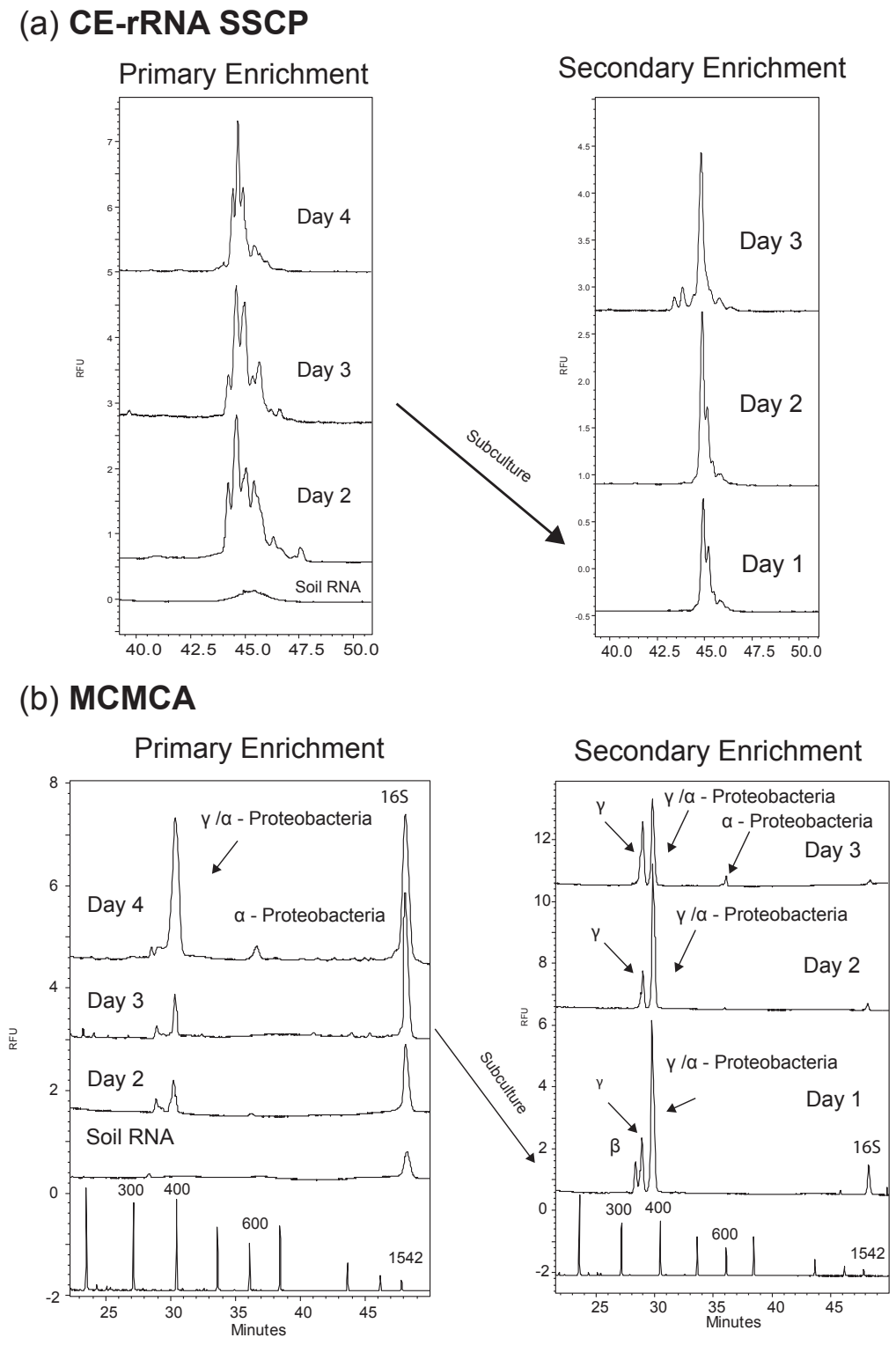

Figure 4-13 Application of the MCMCA and CE-rRNA SSCP method to characterise microbes in actual community sample in primary and secondary hydrocarbon degradation microbes enrichment cultures over time. Electropherograms of (a) CE-rRNA SSCP and (b) MCMCA are shown. Electrophoresis conditions as described in Chapter 3 for CE-rRNA SSCP and in Figure 4-12 for MCMCA. Arrows indicate the present of the targeted microbial groups.

4.4. Concluding remarks

This chapter described the proof of concept of a multiplex RNase H cleavage approach that has the potential to facilitate rapid and easy evaluation of multiple dominant microbes within an active community over time. Yet, the shortcomings of MCMCA approach were gradually revealed in this study. The major shortcoming of the developed method at this stage is its dependence on having a collection of appropriate scissor probes. As demonstrated earlier, the elucidation of the optimal multiplex cleavage reaction condition to provide the necessary stringency represents a search for compromise. Unlike previous reports on single probe cleavage, this study has revealed that previously published oligonucleotide probes (suitable for PCR) may not necessary be suitable for direct application in multiplex cleavage. Issues such as (i) determining a common reaction condition for multiple probes, (ii) stringency adjustment to minimise nonspecific hybridisation on non-target sequences and (iii) low G+C content of scissor probe, each represents challenges that are required to be overcome to achieve the ideal multiplex cleavage with high reliability, specificity and cleavage efficiency. If this can be done, then when combined with a high resolution size-separation, this method may have the potential to monitor 10's or 100's of microbes within a community. Nevertheless, the MCMCA method proposed here was applied to actual ecosystems containing complex microbial communities, and provided interesting data regarding measurements of specific microbial populations based on their rRNA. In conjunction with the previously developed CE-rRNA SSCP approach, the MCMCA method was applied to characterise the rRNA level of different microbial groups in the hydrocarbon degradation enrichment process. In comparison to the currently widely used molecular tools such as FISH and membrane hybridisation which are relatively

laborious and time consuming, the experimental procedures of the MCMCA method are very simple to carry out and can be completed in less than 3 hrs. In this regard, MCMCA with an appropriately optimised probe set has the potential to offer a rapid and easy means of simultaneously linking targeted microbes of interest at different phylogenic levels, as is required in environmental, food, and clinical microbiology, to their corresponding metabolic activity and/or relative abundances.

4.5. References

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GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The following general conclusions can be made from this project relating to the development of a new CE based microbial community characterisation approach that aimed to be simple, cost effective and a 'single method' approach.

Replaceable sieving polymers are the fundamental component for high resolution nucleic acids separation in CE. The choice of the polymer and its physical properties play significant roles in influencing separation performance and are related to its potential applications. RAFT polymerisation was investigated in Chapter 2 as an alternative strategy to conventional free radical polymerisation to synthesize high molecular weight sieving polymer. For the very first time, a well-defined high molecular weight PDMA at 765,000 $gmol^{-1}$ with a PDI of 1.55 was successfully synthesised with the use of chain transfer agent – PABTC in a multi-step sequential RAFT polymerisation approach. This study represents the first demonstration of RAFT polymerisation for synthesising polymers with the molecular weight range necessary for high resolution separation in sieving electrophoresis. Adjustment of pH in the reaction was found to be crucial for the successful RAFT polymerisation of high molecular weight polymer as the buffered condition minimises the effect of hydrolysis and aminolysis commonly associated with trithiocarbonates chain transfer agents. The separation efficiency of PABTC-PDMA was found to have marginally superior separation performance compared to a commercial PDMA (POP™) formulation of similar molecular weight range.

At the time of writing this conclusion, there had been no published demonstration of sieving polymer synthesised by RAFT polymerisation. RAFT polymerisation is best known for its versatility and capability to yield well defined block polymers previously unattainable by conventional free radical polymerisation. Even though this was not explicitly explored this study, it represents a potential research opportunity to realise potential of RAFT polymerisation to synthesis novel, well defined, complex architecture block copolymers with different physical and chemical functionalities. These novel polymers could potentially offer useful applications in sieving electrophoresis as well as other applications not related to separation science such as surface modifications and clinical therapeutic applications. Moreover, recent reviews [1-3] have highlighted the fact that there have been substantial developments in RAFT polymerisation in recent years particularly in the area of novel RAFT agents, as such future research could be carried out on evaluating new RAFT agents capable of offering better polymerisation kinetics and greater tolerance towards hydrolysis and aminolysis, which would play a vital role in simplifying the synthesis procedure.

A novel quantitative CE-based rRNA fingerprinting method, CE-rRNA-SSCP, was presented in Chapter 3 where rRNA fragments were used directly for the construction of microbial profile with no PCR amplification step. The CE-rRNA-SSCP was developed with the aim to obtain a simple RNA fingerprinting technique that would incorporate the advantages of rRNA as a biomarker of microbial taxonomy and dominance. The use of fluorescently labelled hybridisation probes allowed the specific detection of cleaved rRNA fragments. Furthermore, the high sensitivity of CE-LIF significantly decreased the sample requirement as compared to gel based method [4]. The developed approach is not subjected to the amplification bias commonly associated with PCR-based

techniques and its quantitative capability for providing relative RNA abundance information for microbial communities was demonstrated by the metabolic activity shift assay. The use of fluorescently labelled 16S rRNA specific hybridisation probe in the development of CE-rRNA-SSCP has allowed the observation of species-specific signal profiles for the very first time. Similar to a previous study [4], several of the profiles obtained throughout the development showed that a single species was represented by more than one peak in a profile. Although only one peak per species was expected initially, several examples showed that this was not the case. It was hypothesized that the multiple band phenomenon was caused by a combination of factors (e.g. RNA multiplicity and the presence of more than one stable conformational state of a particular RNA sequence under partially denaturing separation conditions). These different shapes may have slightly different electrophoretic mobilities which are detected as different bands in an SSCP profile. As microbial community diversity increases, these multiple peaks began to overlap and consequently cause the deterioration of resolution and the ability to separate different species. This led to the conclusion that the CE-rRNA-SSCP approach developed here has limited utility for environments with high species diversity (richness). Nonetheless in its current state, CE-rRNA-SSCP may be applied to determine community structure, dynamics and stability of enrichment cultures or bioreactors. It could also find use in tracking population changes in biofilms with limited species numbers or environments with expected low species richness and highly specialised microbial groups. To overcome the current limitations, a stronger denaturing separation conditions may be required, and with the use of LNA fluorescently labelled hybridisation probes should solve the detection challenge described in Chapter 3. Alternatively, the resolution limitation could possibly be circumvented by using a single scissor probe targeting a narrower

taxonomic group instead of probe EUB342 (that targets Domain *Bacteria*). i.e. A scissor probe or group of scissor probes could be selected to investigate which microorganisms are present and performing certain task and at the same time, a taxonomic specific probe could be employed to examine if targeted microbial species is actively carrying out the specific task.

In spite of certain limitations highlighted in this thesis, the CE-rRNA-SSCP approach has met the main objectives of the thesis and is a straightforward, fast and cost-effective technique that yields information on the abundance of individual members of microbial communities that is complementary to other existing methods in microbial ecology. The other important matter to address was the ability to link microbial phylogeny to metabolic activity and relative abundance. This was conceptually explored in Chapter 4 with an innovative approach, termed multiplex cleavage microbial community analysis (MCMCA).

MCMCA was designed as a complementary approach to the CE-RNA-SSCP to provide taxonomic information of metabolically active microorganisms and provide information regarding their relative abundance within the microbial community. Instead of using a single universal probe to generate rRNA fragments of similar length, the cleavage reaction of MCMCA is multiplexed by inclusion of a set of microbial group-specific DNA probes that hybridise at different positions on the corresponding rRNA targets. Under stringent hybridisation and RNase H cleavage conditions, 16S RNAs purified from complex communities were cleaved into discrete fragments varying in lengths based on the binding site of the specific scissor probes and were subsequently resolved by size separation in denaturing CE-LIF. The conception and proof of concept of

MCMCA were presented and a potential application for MCMCA was demonstrated in conjunction with CE-rRNA SSCP to characterise the rRNA level of different microbial groups in the community enrichment process. In comparison to the existing molecular tools such as FISH, quantitative PCR and membrane hybridization which are relatively laborious and time consuming, the experimental procedures of the MCMCA method were demonstrated to be fast and relatively straightforward. MCMCA with an appropriately optimised probe set has the potential to offer a rapid and easy means of simultaneously linking targeted microbes of interest at different phylogenic levels to their corresponding metabolic activity and/or relative abundances. Nevertheless, MCMCA still requires extensive optimisation to overcome major obstacles to realise its full potential. As discussed in Chapter 4, the elucidation of the optimal multiplex cleavage reaction conditions with the necessary hybridisation-cleavage stringency represents a challenging task as well as a constant search for compromise. Unlike previous studies on single probe cleavage, oligonucleotide probes published for PCR based approached were discovered to be unsuitable for direct application in multiplex cleavage as the hybridisation process was confounded by the fact that each probe has different hybridisation specificity and thermal stability. However, it is possible to make MCMCA a practical method if the technical challenges highlighted in Chapter 4 can be overcome. The author believes the success of MCMCA method depends on the re-design of specific scissor probe sets with high reliability and specificity. The influx of new rRNA sequence information to the databases may eventually require a re-evaluation of scissor probe specificity and even redesign of probes that can no longer be regarded specific as demonstrated in the development of MCMCA. Additionally, future work in the area of optimising multiplex cleavage reaction with high cleavage efficiency under one common reaction condition would likely require standardisation of

the probe-to-rRNA thermal duplex stability across the candidate probes and this could be potentially achievable with the incorporation of LNA the scissor probe. Moreover, the re-optimisation of the hybridisation-cleavage buffer also represents a great potential research opportunity for future studies. The incorporation of K^+ and NH_4^+ salts in commercial multiplex PCR buffer have been able to maintain high annealing specificity and suppressed binding of primers to mismatch sequences [5]. Alternatively, construction several competitor oligonucleotide probes [6] could also be a potential research opportunity to increase the specificity of scissor probes that show only one mismatch with non-target sequences between closely related organisms. Future work on this strategy should focus on designing competitor probes to perfectly match with non-target sequence at the homologous site and subsequently synthesised with alternative nucleic acid analogues such as peptide nucleic acid (PNA) and 2'-O-alkyl RNA. During hybridisation, these PNA/2'-O-alkyl RNA competitors would provide protection for non-target sequence from being cleaved as they cannot activate RNase H [7].

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Appendix

Synthesis of fluorescently labelled size standard

In house custom size standard primers were designed from sequence of the pCR4-TOPO (hereafter pCR4) vector (Invitrogen, CA, USA). A single forward primer was designed from the T3 priming site with the 6-FAM dye attached to the 5′ end (Sigma Aldrich, Castle Hill, Australia). From the same vector sequence, 10 unlabelled reverse primers (Integrated DNA Technologies, Coralville, USA) were designed using Primer3 [1]1 to yield 103, 200, 300, 400, 505, 600, 700, 1000, 1250 and 1542 bp fragments when used in combination with the T3 forward primer (see Table A1 for primer sequences). 0.5 µL of pCR4 (10 ng/uL stock from TOPO® TA Cloning® Kit reagent) were used as template in 10 separate 25 µL polymerase chain reactions (each with a different reverse primer), containing: 1x Immomix Reagent (Bioline, Alexandria, Australia), 0.8 µM T3-FAM forward primer, 0.8 µM reverse primer and appropriate amount of nuclease free water. Cycling conditions were 35 cycles of 30 s denaturation at 94 ºC, 30 s annealing at 50 ºC (47 ºC for 103R) and 30 s extension at 72 ºC, preceded by a pre-heating step of 10 min at 95 ºC, and terminated by a final extension step of 10 min at 72 ºC. Each PCR product was purified using commercial PCR Purification kit (Norgen Biotek, Thorold, Canada) according to manufacturer instructions and quantified on a Nanodrop 8000 (Thermo Fisher Scientific, Massachusetts, USA). Finally, the purified PCR products were pooled together at approximate equal amounts.

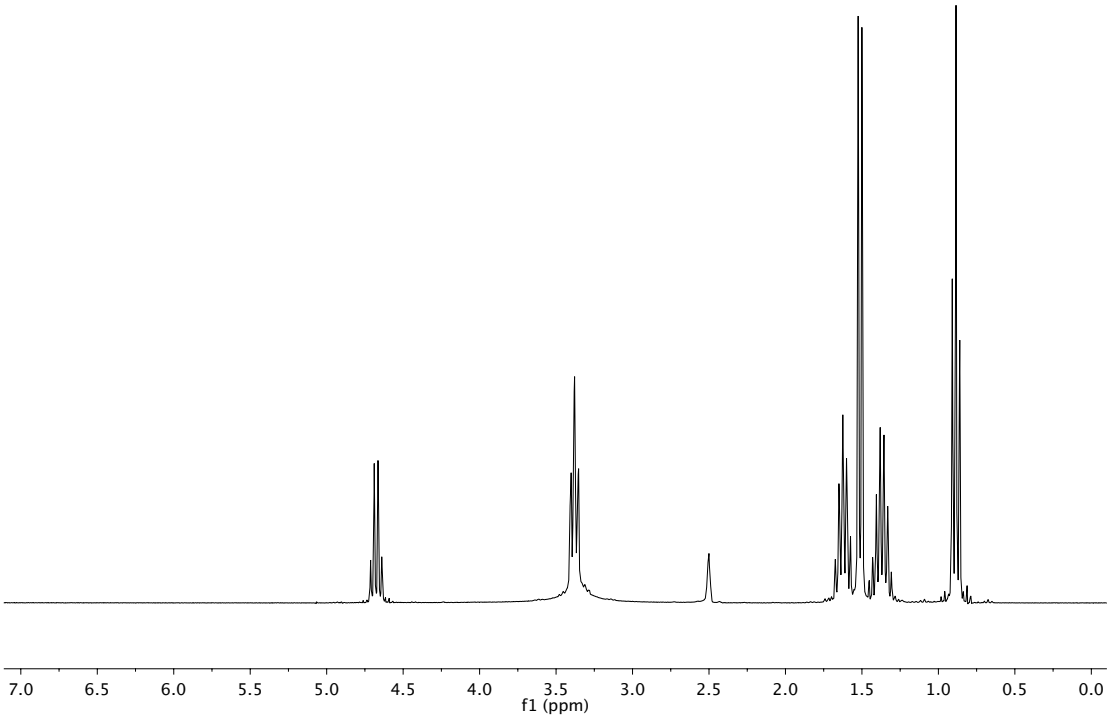
Table A1 Primer Sequences Complementary to pCR4-TOPO vector

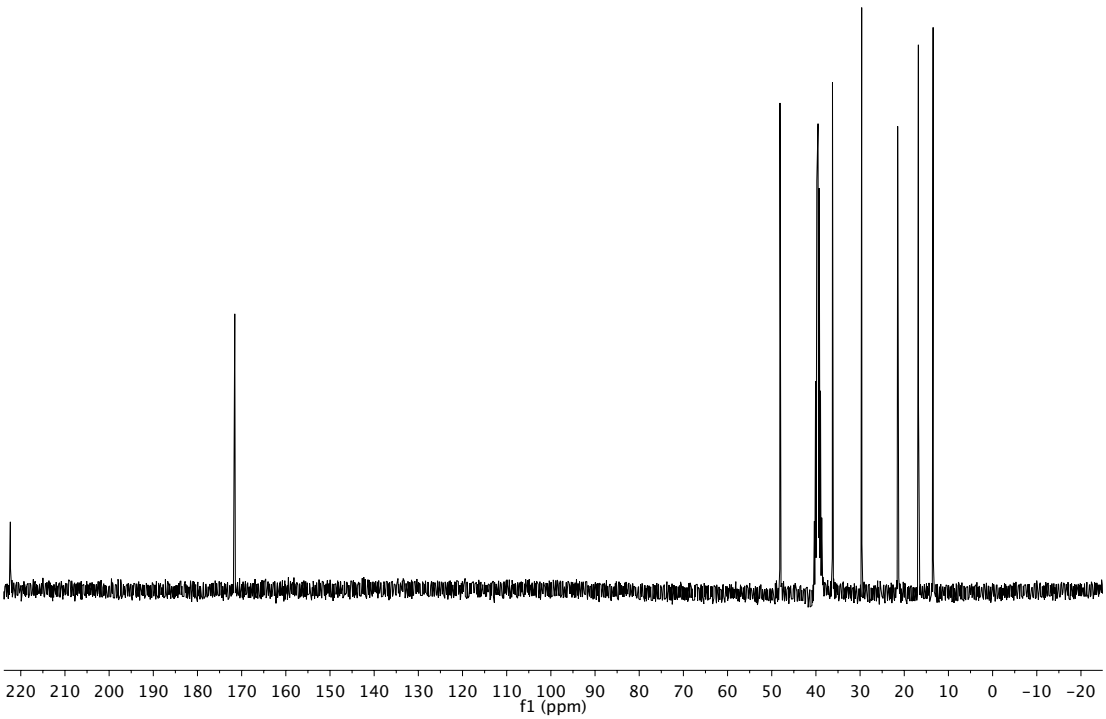
Primer Name	Direction	Primer Sequence (5' - 3')
T3F-FAM	Forward	FAM-AATTAACCCTCACTAAAGGG
pCR4-103R	Reverse	TACGACTCACTATAGGGCGAAT
pCR4-200R	Reverse	GCTGGCGAAAGGGGGATGTG
pCR4-300R	Reverse	GGCTCTCTCTTTTATAGGTG
pCR4-400R	Reverse	ACTTTATCTGACAGCAGACG
pCR4-505R	Reverse	GCCACTTCTTCCCCGATAAC
pCR4-600R	Reverse	AGATCCTTTTTGATAATCTCATGC
pCR4-700R	Reverse	CTTGCGTTTTCCCTTGTCC
pCR4-1000R	Reverse	GTCTGTTGTGCCCAGTCATAG
pCR4-1250R	Reverse	GGCAGGAGCAAGGTGAGAT
pCR4-1542R	Reverse	CCACAGTCGATGAATCCAGA

NMR Spectra of 2-propanoic acid butyl trithiocarbonate (PABTC)

^{1}HNMR (d^{6}-acetone, 300MHz) δ 4.68 (q, 7.14Hz, 1H), 3.39 (t, 7.47Hz, 2H), 1.67-1.58 (m, 2H), 1.51 (d, 7.36Hz, 3H), 1.43-1.31 (m, 2H), 0.88 (t, 7.34Hz, 3H).

^{13}CNMR (d^{6}-acetone, 300MHz) δ 222.4, 171.6, 48.1, 36.3, 29.6, 21.5, 16.8, 13.5.





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